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(54) Title: METHOD FOR PURIFYING HEMOGLOBIN			
(57) Abstract			
<p>A method for producing a purified hemoglobin product includes loading a hemoglobin solution onto an anion exchange chromatography column. At least one tris(hydroxymethyl) aminomethane acetate buffer solution is injected into the column. The buffer solution has a pH lower than that of the column, whereby a purified hemoglobin product elutes from the column. In one embodiment, the hemoglobin solution initially can be equilibrated at a pH of greater than about 8.7. In another embodiment, contaminants can be removed by equilibrating the column with at least about eleven column void volumes of buffer solution at an intermediate pH of between about 8.2 and about 8.6, to thereby form a stepped pH gradient. In still another embodiment, all buffer solutions employed are tris(hydroxymethyl) aminomethane acetate.</p>			

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METHOD FOR PURIFYING HEMOGLOBIN**RELATED APPLICATIONS**

This application is a Continuation of U.S.S.N. 09/113,953, filed July 10, 1998, which is a Continuation-in-Part of U.S.S.N. 08/473,497, filed June 7, 1995, which is a Continuation-in-Part of U.S.S.N. 08/458,916, filed June 2, 1995, which is a Continuation of U.S.S.N. 08/409,337, filed March 23, 1995, the teachings of which are incorporated herein by reference.

10 BACKGROUND OF THE INVENTION

There exists a need for a blood-substitute to treat or prevent hypoxia resulting from blood loss (e.g., from acute hemorrhage or during surgical operations), resulting from anemia (e.g., pernicious anemia or sickle cell anemia), or resulting from shock (e.g., volume deficiency shock, anaphylactic shock, septic shock or allergic shock). The use of blood and blood fractions as in these capacities as a blood-substitute is fraught with disadvantages. For example, the use of whole blood often is accompanied by the risk of transmission of hepatitis-producing viruses and AIDS-producing viruses which can complicate patient recovery or result in patient fatalities. Additionally, the use of whole blood requires blood-typing and cross-matching to avoid immunohematological problems and interdonor incompatibility.

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Human hemoglobin, as a blood-substitute, possesses osmotic activity and the ability to transport and transfer oxygen, but it has the disadvantage of rapid elimination from circulation by the renal route and through vascular walls, resulting in a very short, and therefore, a typically unsatisfactory half-life. Further, human hemoglobin is also frequently contaminated with toxic levels of endotoxins, bacteria and/or viruses.

Non-human hemoglobin suffers from the same deficiencies as human hemoglobin. In addition, hemoglobin from non-human sources is also typically contaminated with proteins, such as antibodies, which could cause an immune system response in the recipient.

Previously, at least four other types of blood-substitutes have been utilized, including perfluorochemicals, synthesized hemoglobin analogues, liposome-encapsulated hemoglobin, and chemically-modified hemoglobin. However, many of these blood-substitutes have typically had short intravascular retention times, being removed by the circulatory system as foreign substances or lodging in the liver, spleen, and other tissues. Also, many of these blood-substitutes have been biologically incompatible with living systems.

SUMMARY OF THE INVENTION

The invention relates to a method for producing a purified hemoglobin product.

In one embodiment, the method includes loading a hemoglobin solution onto an anion exchange chromatography column. At least one tris(hydroxymethyl) aminomethane acetone buffer solution is injected into the column, the buffer solution having a pH lower than that of the column,

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whereby a purified hemoglobin products elutes from the column.

In another embodiment, a hemoglobin solution is loaded onto an anion exchange column, the column initially being
5 equilibrated to a pH greater than about 8.7. At least one buffer solution then is injected into the column, the buffer solution having a pH that is lower than about 8.6, whereby the purified hemoglobin product elutes from the column.

10 Still another embodiment includes loading a hemoglobin solution onto an anion exchange chromatography column. At least eleven column void volumes of equilibrating buffer solution, having a pH in a range of between about 8.2 and about 8.6, are then injected into the column. A buffer
15 solution having a pH lower than that of the equilibrating buffer solution then is injected into the column, whereby a purified hemoglobin product elutes from the column.

In yet another embodiment, a hemoglobin solution is loaded onto an anion exchange chromatography column that
20 has been initially calibrated to a pH greater than about 8.7. At least eleven column void volumes of an equilibrating buffer solution of tris(hydroxymethyl) aminomethane acetate, having a pH in a range of between about 8.2 and about 8.6, are then injected into the column.
25 A buffer solution of tris(hydroxymethyl) aminomethane acetate, having a pH lower than about 8.2, then is injected into the column, whereby the purified hemoglobin solution elutes from the column.

The method of this invention advantageously achieves a
30 hemoglobin product that is substantially free of even recalcitrant protein materials such as carbonic anhydrase. The method can also obtain a relatively high yield of

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hemoglobin from a solution that includes many contaminants. Thus, the hemoglobin derived from one species can be successfully used in a different species as a blood-substitute without the recipient species suffering
5 significant side effects.

DETAILED DESCRIPTION OF THE INVENTION

The features and other details of the process of the invention will now be more particularly described with reference to the accompanying drawings and pointed out in
10 the claims. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as limitations of the invention. The principle features of this invention can be employed in various embodiments without departing from the scope of the
15 present invention.

The invention relates to a method for producing a purified hemoglobin product substantially free of other blood protein components and contaminants, employing a chromatographic column. The method is characterized in the
20 use of a pH gradient to elute the hemoglobin component.

Concentrated Hb solution obtained from the disruption, fractionation and/or ultrafiltration of red blood cells, is directed into one or more parallel chromatographic columns to further separate the hemoglobin by high performance
25 liquid chromatography from other contaminants such as antibodies, endotoxins, phospholipids, enzymes (such as, carbonic anhydrase), viruses and transmissible spongiform encephalopathy agents. The chromatographic column contains an anion exchange medium suitable to separate Hb from
30 non-hemoglobin proteins. Suitable anion exchange media include, for example, silica, alumina, titania gel, cross-

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linked dextran, agarose or a derivatized moiety, such as a polyacrylamide, a polyhydroxyethyl-methacrylate or a styrene divinylbenzene, that has been derivatized with a cationic chemical functionality, such as a
5 diethylaminoethyl or quaternary aminoethyl group. A suitable anion exchange medium and corresponding eluants for the selective absorption and desorption of Hb as compared to other proteins and contaminants, which are likely to be in a lysed RBC phase, are readily determinable
10 by one of reasonable skill in the art.

In a more preferred embodiment, a method is used to form an anion exchange medium from silica gel which is hydrothermally treated to increase the pore size, exposed to γ -glycidoxyl propylsilane to form active epoxide groups
15 and then exposed to $C_3H_7(CH_3)_2NCl$ to form a quaternary ammonium anion exchange medium. This method is described in the *Journal of Chromatography*, 120:321-333 (1976), which is incorporated herein by reference in its entirety. In one embodiment, the chromatographic column, or columns, are
20 first equilibrated to a pH greater than about 8.7.

Preferably, the chromatography column is equilibrated to a pH in a range of between about 8.7 and about 10.0. In a particularly preferred embodiment, the pH of equilibration is in a range of between about 8.7 and about 9.3 and, most
25 preferably, in a range of between about 8.9 and about 9.1. Preferably, the buffer employed to initially equilibrate the column is tris(hydroxymethyl) aminomethane acetate (Tris-acetate), and has a concentration of about 20 mmoles/liter (mM/l).

30 Hemoglobin solution that, preferably, has been dialyzed against purified water (U.S.P.) and, also preferably, has a conductivity of about 280 μ S/cm and a pH

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between about 6.75 and about 7.75, is then injected onto the medium in the column to thereby load the column with hemoglobin. The concentration of hemoglobin solution that is loaded onto the column typically has a concentration in 5 a range of between about 90 and about 200 grams/liter. Preferably, the concentration of the hemoglobin solution is in a range of between about 90 and about 110 grams/liter. Preferably, after injecting the concentrated Hb solution, the chromatographic column is washed for about ten minutes 10 (4 column void volumes) with the Tris-acetate to elute non-hemoglobin components that do not bind to the media, and to facilitate strong bonding of hemoglobin to the media.

A pH gradient is used in the chromatographic column to separate protein contaminants, such as the enzyme carbonic 15 anhydrase, phospholipids, antibodies and endotoxins, from the Hb. The pH gradient can be a continuous gradient or a stepped gradient. Buffer solutions having different pH values are sequentially injected into the column to create a pH gradient of the eluate over time. It is preferred 20 that the buffers be filtered prior to injection, such as with a suitable 10,000 Dalton depyrogenation membrane. The buffer solutions should be of monovalent buffers which have a low ionic strength so that elution of Hb and non- 25 hemoglobin contaminants is generally dependent upon pH and not significantly dependent upon ionic strength.

Typically, buffers with an ionic concentration of about 50 mM, or less, have suitably low ionic strengths.

Preferably, the contaminants and hemoglobin of the 30 hemoglobin solution are separated by elution from the column in a stepped gradient, whereby a buffer is employed that has a pH between that at which the hemoglobin is loaded onto the column and a final pH, at which hemoglobin

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is eluted from the column. In one embodiment, the stepped gradient includes injecting a suitable buffer solution into the column. The buffer solution has a pH lower than the pH of the column at the time the column initially was loaded 5 with hemoglobin. The column is equilibrated with the buffer solution. Preferably, at least about six column volumes of the buffer solution are injected into the column. A "column volume," as defined herein, is the volume of the column, not including any packing material.

10 Typically, suitable column packings, i.e. exchange media, for use with the present invention, cause the column to have a void fraction of about 0.525 of the total volume of the column. Six column volumes, therefore, is equivalent to about 11.7 "column void volumes." Generally, at least 15 about 11 column void volumes of buffer solution are injected into the column.

Preferably, the pH of the buffer solution is lower than about 8.6. More preferably, the pH of the buffer solution is in a range of between about 8.2 and about 8.4.

20 In an especially preferred embodiment, the buffer solution is tris(hydroxymethyl) aminomethane acetate (Tris-acetate). Contaminants of the hemoglobin solution are eluted from the column by equilibrating the column in this manner.

Thereafter, a buffer is injected into the column to 25 elute hemoglobin. Preferably, the buffer solution is of tris(hydroxymethyl) aminomethane acetate. More preferably, the Tris-acetate solution has a pH in a range of between about 6.5 and about 7.5. The hemoglobin eluate is the purified hemoglobin product.

30 In a preferred embodiment, the first 3%-to-4% of the Hb eluate and the last 3%-to-4% of the Hb eluate are directed to waste to provide assurance of the purity of the

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Hb eluate. It is preferred that the Hb eluate be directed through a sterile filter. Suitable sterile filters include 0.22 µm filters, such as a Sartorius Sartobran Cat # 5232507 G1PH filter.

5 Wherein the chromatographic columns are to be reused, contaminating non-hemoglobin proteins and endotoxin remaining in the columns are then eluted by a fourth buffer. An example of a suitable buffer solution is a NaCl/Tris-acetate solution (concentrations about 1.0 M NaCl
10 and about 20 mM Tris-acetate; pH about 8.4 to about 9.4, preferably about 8.9-9.1). In a most preferred embodiment all of the buffer solutions are of tris(hydroxymethyl) aminomethane acetate. Typically, the buffer solutions used are at a temperature in a range of between about 0°C and
15 about 50°C. Preferably, buffer temperature is about 12.4 ± 1.0°C during use. In addition, the buffers are typically stored at a temperature in a range of between about 9 °C and about
11 °C.

20 As defined herein, a blood-substitute is a hemoglobin-based oxygen carrying composition for use in humans, mammals and other vertebrates, which is capable of transporting and transferring oxygen to vital organs and tissues, at least, and can maintain sufficient
25 intravascular oncotic pressure. A vertebrate is as classically defined, including humans, or any other vertebrate animals which uses blood in a circulatory system to transfer oxygen to tissue. Additionally, the definition of circulatory system is as classically defined, consisting
30 of the heart, arteries, veins and microcirculation including smaller vascular structures such as capillaries.

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A blood-substitute formed by the method of invention preferably is made according to one embodiment of the invention must have levels of endotoxins, phospholipids, foreign proteins and other contaminants which will not
5 result in a significant immune system response and which are non-toxic to the recipient. Preferably, a blood-substitute is ultrapure. "Ultrapure," as defined herein, means containing less than 0.5 EU/ml of endotoxin, less than 3.3 nmoles/ml phospholipids and little to no
10 detectable levels of non-hemoglobin proteins, such as serum albumin or antibodies.

The term "endotoxin" refers to the cell-bound lipopolysaccharides, produced as a part of the outer layer of gram-negative bacterial cell walls, which under many
15 conditions are toxic. When injected into animals, endotoxins can cause fever, diarrhea, hemorrhagic shock, and other tissue damage. Endotoxin unit (EU) has been defined by the United States Pharmacopeial Convention of 1983, page 3014, as the activity contained in 0.1 nanograms
20 of U.S. reference standard lot EC-5. One vial of EC-5 contains 10,000 EU. Examples of suitable means for determining endotoxin concentrations in a blood-substitute include the method "Kinetic/ Turbidimetric Limulus Amebocytic Lystate (LAL) 5000 Methodology" developed by
25 Associates of Cape Cod, Woods Hole, Massachusetts.

"Stable polymerized hemoglobin," as defined herein, is a hemoglobin-based oxygen carrying composition which does not substantially increase or decrease in molecular weight distribution and/or in methemoglobin content during storage
30 periods at suitable storage temperatures for periods of two years or more, and preferably for periods of two years or more, when stored in a low oxygen environment. Suitable

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storage temperatures for storage of one year or more are between about 0 °C and about 40 °C. The preferred storage temperature range is between about 0 °C and about 25 °C.

A suitable low oxygen environment, or an environment
5 that is substantially oxygen-free, is defined as the cumulative amount of oxygen in contact with the blood-substitute, over a storage period of at least about two months, preferably at least about one year, or more preferably at least about two years which will result in a
10 methemoglobin concentration of less than about 15% by weight in the blood-substitute. The cumulative amount of oxygen includes oxygen inleakage into the blood-substitute packaging and the original oxygen content of the blood-substitute and packaging.

15 Throughout this method, from red blood cell (RBC) collection until hemoglobin polymerization, blood solution, RBCs and hemoglobin are maintained under conditions sufficient to minimize microbial growth, or bioburden, such as maintaining temperature at less than about 20 °C and
20 above 0 °C. Preferably, temperature is maintained at a temperature of about 15 °C or less. More preferably, the temperature is maintained at 10 ± 2 °C.

In this method, portions of the components for the process for preparing a stable polymerized hemoglobin
25 blood-substitute are sufficiently sanitized to produce a sterile product. Sterile is as defined in the art, specifically, that the solution meets United States Pharmacopeia requirements for sterility provided in USP XXII, Section 71, pages 1483-1488. Further, portions of
30 components that are exposed to the process stream, are usually fabricated or clad with a material that will not react with or contaminate the process stream. Such

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materials can include stainless steel and other steel alloys, such as Inconel.

Suitable RBC sources include human blood, bovine blood, ovine blood, porcine blood, blood from other 5 vertebrates and transgenically-produced hemoglobin, such as the transgenic Hb described in *BIO/TECHNOLOGY*, 12: 55-59 (1994).

The blood can be collected from live or freshly slaughtered donors. One method for collecting bovine whole 10 blood is described in U.S. Patent Nos. 5,084,558 and 5,296,465, issued to Rausch *et al.* It is preferred that the blood be collected in a sanitary manner.

At or soon after collection, the blood is mixed with at least one anticoagulant to prevent significant clotting 15 of the blood. Suitable anticoagulants for blood are as classically known in the art and include, for example, sodium citrate, ethylenediaminetetraacetic acid and heparin. When mixed with blood, the anticoagulant may be in a solid form, such as a powder, or in an aqueous 20 solution.

It is understood that the blood solution source can be from a freshly collected sample or from an old sample, such as expired human blood from a blood bank. Further, the blood solution could previously have been maintained in 25 frozen and/or liquid state. It is preferred that the blood solution is not frozen prior to use in this method.

In another embodiment, prior to introducing the blood solution to anticoagulants, antibiotic levels in the blood solution, such as penicillin, are assayed. Antibiotic 30 levels are determined to provide a degree of assurance that the blood sample is not burdened with an infecting organism by verifying that the donor of the blood sample was not

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being treated with an antibiotic. Examples of suitable assays for antibiotics include a penicillin assay kit (Difco, Detroit, MI) employing a method entitled "Rapid Detection of Penicillin in Milk". It is preferred that 5 blood solutions contain a penicillin level of less than or equal to about 0.008 units/ml. Alternatively, a herd management program to monitor the lack of disease in or antibiotic treatment of the cattle may be used.

Preferably, the blood solution is strained prior to or 10 during the anticoagulation step, for example by straining, to remove large aggregates and particles. A 600 mesh screen is an example of a suitable strainer.

The RBCs in the blood solution are then washed by suitable means, such as by diafiltration or by a 15 combination of discrete dilution and concentration steps with at least one solution, such as an isotonic solution, to separate RBCs from extracellular plasma proteins, such as serum albumins or antibodies (e.g., immunoglobulins (IgG)). It is understood that the RBCs can be washed in a 20 batch or continuous feed mode.

Acceptable isotonic solutions are as known in the art and include solutions, such as a citrate/saline solution, having a pH and osmolarity which does not rupture the cell membranes of RBCs and which displaces plasma portion of the 25 whole blood. A preferred isotonic solution has a neutral pH and an osmolarity between about 285-315 mOsm. In a preferred embodiment, the isotonic solution is composed of an aqueous solution of sodium citrate dihydrate (6.0 g/l) and of sodium chloride (8.0 g/l).

30 Water which can be used in the method of invention include distilled water, deionized water, water-for-injection (WFI) and/or low pyrogen water (LPW). WFI, which

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is preferred, is deionized, distilled water that meets U.S. Pharmacological Specifications for water-for-injection. WFI is further described in *Pharmaceutical Engineering*, 11, 15-23 (1991). LPW, which is preferred, is deionized water
5 containing less than 0.002 EU/ml.

It is preferred that the isotonic solution be filtered prior to being added to the blood solution. Examples of suitable filters include a Millipore 10,000 Dalton ultrafiltration membrane, such as a Millipore Cat # CDUF
10 050 G1 filter or A/G Technology hollow fiber, 10,000 Dalton (Cat # UFP-10-C-85).

In a preferred embodiment, RBCs in the blood solution are washed by diafiltration. Suitable diafilters include microporous membranes with pore sizes which will separate
15 RBCs from substantially smaller blood solution components, such as a 0.1 μm to 0.5 μm filter (e.g., a 0.2 μm hollow fiber filter, Microgon Krosflo II microfiltration cartridge). Concurrently, filtered isotonic solution is added continuously (or in batches) as makeup at a rate
20 equal to the rate (or volume) of filtrate lost across the diafilter. During RBC washing, components of the blood solution which are significantly smaller in diameter than RBCs, or are fluids such as plasma, pass through the walls of the diafilter in the filtrate. RBCs, platelets and
25 larger bodies of the diluted blood solution, such as white blood cells, are retained and mixed with isotonic solution, which is added continuously or batchwise to form a dialyzed blood solution.

In a more preferred embodiment, the volume of blood
30 solution in the diafiltration tank is initially diluted by the addition of a volume of a filtered isotonic solution to the diafiltration tank. Preferably, the volume of isotonic

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solution added is about equal to the initial volume of the blood solution.

In an alternate embodiment, the RBCs are washed through a series of sequential (or reverse sequential) 5 dilution and concentration steps, wherein the blood solution is diluted by adding at least one isotonic solution, and is concentrated by flowing across a filter, thereby forming a dialyzed blood solution.

RBC washing is complete when the level of plasma 10 proteins contaminating the RBCs has been substantially reduced (typically at least about 90%). Typically, RBC washing is complete when the volume of filtrate drained from diafilter 34 equals about 300%, or more, of the volume of blood solution contained in the diafiltration tank prior 15 to diluting the blood solution with filtered isotonic solution. Additional RBC washing may further separate extracellular plasma proteins from the RBCs. For instance, diafiltration with 6 volumes of isotonic solution may remove at least about 99% of IgG from the blood solution.

20 The dialyzed blood solution is then exposed to means for separating the RBCs in the dialyzed blood solution from the white blood cells and platelets, such as by centrifugation.

It is understood that other methods generally known in 25 the art for separating RBCs from other blood components can be employed. For example, sedimentation, wherein the separation method does not rupture the cell membranes of a significant amount of the RBCs, such as less than about 30% of the RBCs, prior to RBC separation from the other blood 30 components.

Following separation of the RBCs, the RBCs are lysed by a means for lysing RBCs to release hemoglobin from the

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RBCs to form a hemoglobin-containing solution. Lysis means can use various lysis methods, such as mechanical lysis, chemical lysis, hypotonic lysis or other known lysis methods which release hemoglobin without significantly 5 damaging the ability of the Hb to transport and release oxygen.

In yet another embodiment, recombinantly produced hemoglobin, such as the recombinantly produced hemoglobin described in *Nature*, 356: 258-260 (1992), can be processed 10 in the method of invention in place of RBCs. The bacteria cells containing the hemoglobin are washed and separated from contaminants as described above. These bacteria cells are then mechanically ruptured by means known in the art, such as a ball mill, to release hemoglobin from the cells 15 and to form a lysed cell phase. This lysed cell phase is then processed as is the lysed RBC phase.

Following lysis, the lysed RBC phase is then ultrafiltered to remove larger cell debris, such as proteins with a molecular weight above about 100,000 20 Daltons. Generally, cell debris include all whole and fragmented cellular components with the exception of Hb, smaller cell proteins, electrolytes, coenzymes and organic metabolic intermediates. Acceptable ultrafilters include, for example, 100,000 Dalton filters made by Millipore (Cat 25 # CDUF 050 H1) and made by A/G Technology (Needham, MA.; Model No. UFP100E55).

It is preferred that ultrafiltration continues until the concentration of Hb in the lysed RBC phase is less than 8 grams/liter (g/l) to maximize the yield of hemoglobin 30 available for polymerization. Other methods for separating Hb from the lysed RBC phase can be employed, including sedimentation, centrifugation or microfiltration.

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The Hb ultrafiltrate can then be ultrafiltered to remove smaller cell debris, such as electrolytes, coenzymes, metabolic intermediates and proteins less than about 30,000 Daltons in molecular weight, and water from 5 the Hb ultrafiltrate. Suitable ultrafilters include a 30,000 Dalton ultrafilter (Millipore Cat # CDUF 050 T1 and/or Armicon, # 540 430).

The concentrated Hb solution can then be directed into one or more parallel chromatographic columns as described 10 in more detail above.

The Hb eluate obtained from the chromatography step is then preferably deoxygenated prior to polymerization to form a deoxygenated Hb solution (hereinafter deoxy-Hb) by means that substantially deoxygenate the Hb without 15 significantly reducing the ability of the Hb in the Hb eluate to transport and release oxygen, such as would occur from denaturation or formation of oxidized hemoglobin (met Hb).

In one embodiment, the Hb eluate is deoxygenated by 20 gas transfer of an inert gas across a phase membrane. Such inert gases include, for example, nitrogen, argon and helium. It is understood that other means for deoxygenating a solution of hemoglobin, which are known in the art, can be used to deoxygenate the Hb eluate. Such 25 other means, can include, for example, nitrogen sparging of the Hb eluate, chemical scavenging with reducing agents such as N-acetyl-L-cysteine (NAC), cysteine, sodium dithionite or ascorbate, or photolysis by light.

Following elution from the chromatographic column, the 30 Hb eluate is preferably concentrated to improve the efficiency of the process. The Hb eluate is recirculated through an ultrafilter to concentrate the Hb eluate to form

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a concentrated Hb solution. Suitable ultrafilters include, for example, 30,000 or less Dalton ultrafilters (e.g., Millipore Helicon, Cat # CDUF050G1 or Amicon Cat # 540430). Typically, concentration of the Hb eluate is complete when 5 the concentration of Hb is between about 100 to about 120 g/l. While concentrating the Hb eluate, the Hb eluate temperature is preferably maintained at approximately 8-12 °C.

Buffer is then directed into the Hb solution, which is 10 preferably concentrated, to adjust the ionic strength of the Hb solution to enhance Hb deoxygenation. It is preferred that the ionic strength be adjusted to between about 150 meq/l and about 200 meq/l to reduce the oxygen affinity of the Hb in the Hb solution. Suitable buffers 15 include buffers with a pH that will not result in significant denaturing of the Hb protein but will have an ionic strength sufficiently high to promote Hb deoxygenation. Examples of suitable buffers include saline solutions with a pH range of about 6.5 to about 8.9. A 20 preferred buffer is an aqueous 1.0 M NaCl, 20 mM Tris-acetate solution with a pH of about 8.9.

Preferably, the resulting buffered Hb solution is then recirculated through the ultrafilter, to again concentrate the Hb solution to improve the efficiency of the process. 25 In a preferred embodiment, concentration is complete when the concentration of Hb is about 100 g/l to about 120 g/l.

During deoxygenation the Hb solution is circulated through a suitable phase transfer membrane. Appropriate phase transfer membranes include, for example, a 0.05 µm 30 polypropylene hollow fiber microfilter (e.g., Hoechst-Celanese Cat # 5PCM-107). Concurrently, a counterflow of an inert gas is passed across the phase transfer membrane.

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Suitable inert gases include, for example, nitrogen, argon and helium. Gas exchange across phase transfer membrane thereby strips oxygen out of the Hb solution.

Deoxygenation continues until the pO₂ of the Hb solution is reduced to a level wherein the oxygenated Hb (oxyhemoglobin or HbO₂) content in the Hb solution is about 20% or less. In a preferred embodiment, the HbO₂ content in the Hb solution is about 10% or less.

During deoxygenation, the temperature of the Hb solution is typically maintained at a level that will balance the rate of deoxygenation against the rate of methemoglobin formation. Temperature is maintained to limit methemoglobin content to less than 20%. An optimum temperature will result in less than about 5% methemoglobin content, and preferably less than about 2.5% methemoglobin content, while still deoxygenating the Hb solution.

Typically, during deoxygenation the temperature of the Hb solution is maintained between about 19 °C and about 31 °C.

During deoxygenation, and subsequently throughout the remaining steps of the method of invention, the Hb is maintained in a low oxygen environment to minimize oxygen absorption by the Hb and to maintain an HbO₂ content of less than about 20%, preferably less than about 10%.

The deoxygenated-Hb is then preferably equilibrated with a low oxygen content storage buffer, containing a sulfhydryl compound, to form an oxidation-stabilized deoxy-Hb. Suitable sulfhydryl compounds include non-toxic reducing agents, such as N-acetyl-L-cysteine (NAC) D,L-cysteine, γ-glutamyl-cysteine, glutathione, 2,3-dimercapto-1-propanol, 1,4-butanedithiol, thioglycolate, and other biologically compatible sulfhydryl compounds. The oxygen content of a low oxygen content storage buffer must be low

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enough not to significantly reduce the concentration of sulfhydryl compound in the buffer and to limit oxyhemoglobin content in oxidation stabilized deoxy-Hb to about 20% or less, preferably less than about 10%.

5 Typically, the storage buffer has a pO₂ of less than about 50 torr.

In a preferred embodiment, the storage buffer should have a pH suitable to balance Hb polymerization and methemoglobin formation, typically between about 7.6 and 10 about 7.9.

The amount of a sulfhydryl compound mixed with the deoxy-Hb is an amount high enough to increase intramolecular cross-linking of Hb during polymerization and low enough not to significantly decrease intermolecular 15 cross-linking of Hb molecules, due to a high ionic strength. Typically, about one mole of sulfhydryl functional groups (-SH) are needed to oxidation stabilize between about 0.25 moles to about 5 moles of deoxy-Hb.

In a preferred embodiment, the storage buffer contains 20 approximately 25-35 mM sodium phosphate buffer (pH 7.7-7.8) and contains an amount of NAC such that the concentration of NAC in oxidation stabilized deoxy-Hb is between about 0.003% and about 0.3%, by weight. More preferably, the NAC concentration in the oxidation stabilized deoxy-Hb is 25 between about 0.05% and about 0.2% by weight.

Preferably, the storage buffer is filtered prior to mixing with the deoxy-Hb, such as through a 10,000 Dalton ultrafiltration membrane (Millipore Helicon Cat # CDUF050G1 or A/G Technology Maxcell Cat # UFP-10-C-75).

30 In one embodiment, the oxidation-stabilized deoxy-Hb then flows through an optional filter. Suitable filters include a 0.2 µm polypropylene prefilter and a 0.5 µm

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sterile microfilter (Pall Profile II, Cat # ABIY005Z7 or Gelman Supor). The deoxy-Hb is maintained under a substantially oxygen-free atmosphere. This can be accomplished, for example, by purging and blanketing the 5 process apparatus with an inert gas, such as nitrogen, prior to and after filling with oxidation-stabilized deoxy-Hb.

Optionally, prior to transferring the oxidation-stabilized deoxy-Hb to polymerization, an appropriate 10 amount of water is added to the polymerization reactor. In one embodiment an appropriate amount of water is that amount which would result in a solution with a concentration of about 10 to about 100 g/l Hb when the oxidation-stabilized deoxy-Hb is added to the 15 polymerization reactor. Preferably, the water is oxygen-depleted.

After the pO₂ of the water in the polymerization step is reduced to a level sufficient to limit HbO₂ content to about 20%, typically less than about 50 torr, the 20 polymerization reactor is blanketed with an inert gas, such as nitrogen. The oxidation-stabilized deoxy-Hb is then transferred into the polymerization reactor, which is concurrently blanketed with an appropriate flow of an inert gas.

25 The temperature of the oxidation-stabilized deoxy-Hb solution in polymerization reactor is raised to a temperature to optimize polymerization of the oxidation-stabilized deoxy-Hb when contacted with a cross-linking agent. Typically, the temperature of the oxidation- 30 stabilized deoxy-Hb is about 25 °C to about 45 °C, and preferably about 41 °C to about 43 °C throughout polymerization. An example of an acceptable heat transfer

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means for heating the polymerization reactor is a jacketed heating system which is heated by directing hot ethylene glycol through the jacket.

The oxidation-stabilized deoxy-Hb is then exposed to a
5 suitable cross-linking agent at a temperature sufficient to polymerize the oxidation-stabilized deoxy-Hb to form a solution of polymerized hemoglobin (poly(Hb)) over a period of about 2 hours to about 6 hours.

Examples of suitable cross-linking agents include
10 polyfunctional agents that will cross-link Hb proteins, such as glutaraldehyde, succindialdehyde, activated forms of polyoxyethylene and dextran, α -hydroxy aldehydes, such as glycolaldehyde, N-maleimido-6-aminocaproyl-(2'-nitro, 4'-sulfonic acid)-phenyl ester, m-maleimidobenzoic acid-N-
15 hydroxysuccinimide ester, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, m-maleimidobenzoyl-N-hydroxysuccinimide ester, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester, N-
20 succinimidyl(4-iodoacetyl)aminobenzoate, sulfosuccinimidyl(4-iodoacetyl)aminobenzoate, succinimidyl 4-(p-maleimidophenyl)butyrate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, N,N'-
25 phenylene dimaleimide, and compounds belonging to the bis-imidate class, the acyl diazide class or the aryl dihalide class, among others.

A suitable amount of a cross-linking agent is that amount which will permit intramolecular cross-linking to
30 stabilize the Hb and also intermolecular cross-linking to form polymers of Hb, to thereby increase intravascular retention. Typically, a suitable amount of a cross-linking

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agent is that amount wherein the molar ratio of cross-linking agent to Hb is in excess of about 2:1. Preferably, the molar ratio of cross-linking agent to Hb is between about 20:1 to 40:1.

5 Preferably, the polymerization is performed in a buffer with a pH between about 7.6 to about 7.9, having a chloride concentration less than or equal to about 35 mmolar.

10 In a preferred embodiment, a suitable amount of the cross-linking agent is added to the oxidation-stabilized deoxy-Hb which are then mixed by a means for mixing with low shear. A suitable low-shear mixing means includes a static mixer. A suitable static mixer is, for example, a "Kenics" static mixer obtained from Chemineer, Inc.

15 In one embodiment, recirculating the oxidation-stabilized deoxy-Hb and the cross-linking agent through the static mixer causes turbulent flow conditions with generally uniform mixing of the cross-linking agent with the oxidation-stabilized deoxy-Hb thereby reducing the potential for forming pockets of deoxy-Hb containing high concentrations of the cross-linking agent. Generally uniform mixing of the cross-linking agent and the deoxy-Hb reduces the formation of high molecular weight Hb polymers, i.e. polymers weighing more than 500,000 Daltons, and also permits faster mixing of the cross-linking agent and the deoxy-Hb during polymerization. Furthermore, significant Hb intramolecular cross-linking will result during Hb polymerization due to the presence of a sulphydryl compound, preferably NAC. While the exact mechanism of the interaction of the sulphydryl compound with glutaraldehyde and/or Hb is not known, it is presumed that the sulphydryl compound affects Hb/cross-linking agent chemical bonding in

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a manner that at least partially inhibits the formation of high molecular weight Hb polymers and preferentially forms stabilized tetrameric Hb.

Poly(Hb) is defined as having significant
5 intramolecular cross-linking if a substantial portion
(e.g., at least about 50%) of the Hb molecules are
chemically bound in the poly(Hb), and only a small amount,
such as less than about 15% are contained within high
molecular weight polymerized hemoglobin chains. High
10 molecular weight poly(Hb) molecules are molecules, for
example, with a molecular weight above about 500,000
Daltons.

In a preferred embodiment, glutaraldehyde is used as
the cross-linking agent. Typically, about 10 to about 70
15 grams of glutaraldehyde are used per kilogram of oxidation-
stabilized deoxy-Hb. More preferably, glutaraldehyde is
added over a period of five hours until approximately 29-31
grams of glutaraldehyde are added for each kilogram of
oxidation-stabilized deoxy-Hb.

20 After polymerization, the temperature of the poly(Hb)
solution in polymerization reactor is typically reduced to
about 15 °C to about 25 °C.

Wherein the cross-linking agent used is not an
aldehyde, the poly(Hb) formed is generally a stable
25 poly(Hb). Wherein the cross-linking agent used is an
aldehyde, the poly(Hb) formed is generally not stable until
mixed with a suitable reducing agent to reduce less stable
bonds in the poly(Hb) to form more stable bonds. Examples
of suitable reducing agents include sodium borohydride,
30 sodium cyanoborohydride, sodium dithionite, trimethylamine,
t-butylamine, morpholine borane and pyridine borane. Prior
to adding the reducing agent, the poly(Hb) solution is

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optionally concentrated by ultrafiltration until the concentration of the poly(Hb) solution is increased to between about 75 and about 85 g/l. An example of a suitable ultrafilter is a 30,000 Dalton filter (e.g., Millipore 5 Helicon, Cat # CDUF050LT and Amicon, Cat # 540430).

The pH of the poly(Hb) solution is then adjusted to the alkaline pH range to preserve the reducing agent and to prevent hydrogen gas formation, which can denature Hb during the subsequent reduction.

10 In one embodiment, the pH is adjusted to greater than 10. The pH can be adjusted by adding a buffer solution to the poly(Hb) solution during or after polymerization. The poly(Hb) is typically purified to remove non-polymerized hemoglobin. This can be accomplished by dialfiltration or 15 hydroxyapatite chromatography (see, e.g. copending U.S. 5,691,453, issued on November 25, 1997, the teachings of which are incorporated herein by reference).

Following pH adjustment, at least one reducing agent, preferably a sodium borohydride solution, is added to the 20 polymerization step typically through the deoxygenation loop. Typically, about 5 to about 18 moles of reducing agent are added per mole of Hb tetramer (per 64,000 Daltons of Hb) within the poly(Hb). In a preferred embodiment, for every nine liters of poly(Hb) solution in polymerization 25 subsystem 98, one liter of 0.25 M sodium borohydride solution is added at a rate of 0.1 to 0.12 lpm.

The pH and electrolytes of the stable poly(Hb) can then be restored to physiologic levels to form a stable 30 polymerized hemoglobin blood-substitute, by diafiltering the stable poly(Hb) with a diafiltration solution having a suitable pH and physiologic electrolyte levels.

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Preferably, the diafiltration solution is a buffer solution.

Wherein the poly(Hb) was reduced by a reducing agent, the diafiltration solution has an acidic pH, preferably 5 between about 4 to about 6.

A non-toxic sulfhydryl compound can also be added to the stable poly(Hb) solution as an oxygen scavenger to enhance the stability of the final polymerized hemoglobin blood-substitute. The sulfhydryl compound can be added as 10 part of the diafiltration solution and/or can be added separately. An amount of sulfhydryl compound is added to establish a sulfhydryl concentration which will scavenge oxygen to maintain methemoglobin content less than about 15% over the storage period. Preferably, the sulfhydryl 15 compound is NAC. Typically, the amount of sulfhydryl compound added is an amount sufficient to establish a sulfhydryl concentration between about 0.05% and about 0.2% by weight.

In a preferred embodiment, the blood-substitute is 20 packaged under aseptic handling conditions while maintaining pressure with an inert, substantially oxygen-free atmosphere, in the polymerization reactor and remaining transport apparatus.

The specifications for a suitable stable polymerized 25 hemoglobin blood-substitute formed by the method of invention are provided in Table I.

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Table I

PARAMETER	RESULTS
pH (18-22°C)	Physiologically acceptable
Endotoxin	Physiologically acceptable
Sterility Test	Meets Test
Phospholipids ^a	Physiologically acceptable
Total Hemoglobin	10 - 250 g/l
Methemoglobin	<15%
Oxyhemoglobin	<10%
Sodium, Na ⁺	Physiologically acceptable
Potassium, K ⁺	
Chloride, Cl ⁻	
Calcium, Ca ⁺⁺	
Boron	
Glutaraldehyde	Physiologically acceptable
N-acetyl-L-cysteine	Physiologically Acceptable
M.W. >500,000	≤15%
M.W. ≤ 65,000	<10%
M.W. <32,000	<5%
Particulate Content >10μ	<12/ml
Particulate Content >25μ	<2/ml

a - measured in Hb before polymerization

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The stable blood-substitute is then stored in a short-term storage container or into sterile storage containers, each having a low oxygen environment as described in detail above. The storage container should also be sufficiently impermeable to water vapor passage to prevent significant concentration of the blood-substitute by evaporation over the storage period. Significant concentration of the blood-substitute is concentration resulting in one or more parameters of the blood-substitute being high out of specification.

The synthesis of a stable polymerized hemoglobin blood-substitute, formed according to the method of invention, is further described in U.S. Patent No. 5,296,465.

Vertebrates which can receive the blood-substitute, formed by the methods of the invention include mammals, such as a human, non-human primate, a dog, a cat, a rat, a horse or a sheep. Further, vertebrates, which can receive said blood-substitute, includes fetuses (prenatal vertebrate), post-natal vertebrates, or vertebrates at time of birth.

A blood-substitute of the present invention can be administered into the circulatory system by injecting the blood-substitute directly and/or indirectly into the circulatory system of the vertebrate, by one or more injection methods. Examples of direct injection methods include intravascular injections, such as intravenous and intra-arterial injections, and intracardiac injections. Examples of indirect injection methods include intraperitoneal injections, subcutaneous injections, such that the blood-substitute will be transported by the lymph system into the circulatory system, injections into the bone marrow by means of a trocar or catheter. Preferably, the blood-substitute is administered intravenously.

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The vertebrate being treated can be normovolemic, hypervolemic or hypovolemic prior to, during, and/or after infusion of the blood-substitute. The blood-substitute can be directed into the circulatory system by methods such as

5 top loading and by exchange methods.

A blood-substitute can be administered therapeutically, to treat hypoxic tissue within a vertebrate resulting from many different causes including reduced RBC flow in a portion of, or throughout, the

10 circulatory system, anemia and shock. Further, the blood-substitute can be administered prophylactically to prevent oxygen-depletion of tissue within a vertebrate, which could result from a possible or expected reduction in RBC flow to a tissue or throughout the circulatory system of the

15 vertebrate. Further discussion of the administration of hemoglobin to therapeutically or prophylactically treat hypoxia, particularly from a partial arterial obstruction or from a partial blockage in microcirculation, and the dosages used therein, is provided copending U.S. Patent

20 Application Serial No. 08/409,337, filed March 23, 1995, which is incorporated herein by reference in its entirety.

Typically, a suitable dose, or combination of doses of blood-substitute, is an amount which when contained within the blood plasma will result in a total hemoglobin

25 concentration in the vertebrate's blood between about 0.1 to about 10 grams Hb/dl, or more, if required to make up for large volume blood losses.

The invention will now be further and specifically described by the following examples.

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EXAMPLE 1

SYNTHESIS OF STABLE POLYMERIZED Hb BLOOD-SUBSTITUTE

As described in U.S. Patent No. 5,296,465, samples of bovine whole blood were collected, mixed with a sodium citrate anticoagulant to form a blood solution, and then analyzed for endotoxin levels.

Each blood solution sample was maintained after collection at a temperature of about 2 °C and then strained to remove large aggregates and particles with a 600 mesh screen.

Prior to pooling, the penicillin level in each blood solution sample was assayed with an assay kit purchased from Difco, Detroit, Michigan using the method entitled "Rapid Detection of Penicillin in Milk" to ensure that penicillin levels in the blood solutions were \leq 0.008 units/ml.

The blood solution samples were then pooled and mixed with depyrogenated aqueous sodium citrate solution to form a 0.2% by weight solution of sodium citrate in bovine whole blood (hereafter "0.2% sodium citrate blood solution").

The 0.2% sodium citrate blood solution was then passed, in-series, through 800 μm and 50 μm polypropylene filters to remove large blood solution debris of a diameter approximately 50 μm or more.

The RBCs were then washed to separate extracellular plasma proteins, such as BSA or IgG, from the RBCs. To wash the RBCs contained in the blood solution, the volume of blood solution in the diafiltration tank was initially diluted by the addition of an equal volume of a filtered isotonic solution to diafiltration tank. The isotonic solution was filtered with a Millipore (Cat # CDUF 050 G1) 10,000 Dalton ultrafiltration membrane. The isotonic solution was composed of 6.0 g/l sodium citrate dihydrate

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and 8.0 g/l sodium chloride in water-for-injection (WFI).

The diluted blood solution was then concentrated back to its original volume by diafiltration through a 0.2 µm hollow fiber (Microgon Krosflo II microfiltration

5 cartridge) diafilter. Concurrently, filtered isotonic solution was added continuously, as makeup, at a rate equal to the rate of filtrate loss through the 0.2 µm diafilter. During diafiltration, components of the diluted blood solution which were significantly smaller in diameter than
10 RBCs, or are fluids such as plasma, passed through the walls of the 0.2 µm diafilter with the filtrate. RBCs, platelets and larger bodies of the diluted blood solution, such as white blood cells, were retained with continuously-added isotonic solution to form a dialyzed blood solution.

15 During RBC washing, the diluted blood solution was maintained at a temperature between approximately 10 to 25 °C with a fluid pressure at the inlet of the diafilter between about 25 psi and about 30 psi to improve process efficiency.

20 RBC washing was complete when the volume of filtrate drained from the diafilter equaled about 600% of the volume of blood solution prior to diluting with filtered isotonic solution.

25 The dialyzed blood solution was then continuously pumped at a rate of approximately 4 lpm to a Sharples Super Centrifuge, Model # AS-16, fitted with a #28 ringdam. The centrifuge was operating while concurrently being fed dialyzed blood solution, to separate the RBCs from the white blood cells and platelets. During operation, the
30 centrifuge rotated at a rate sufficient to separate the RBCs into a heavy RBC phase, while also separating a substantial portion of the white blood cells (WBCs) and platelets into a light WBC phase, specifically about 15,000 rpm. A fraction of the RBC phase and of the WBC phase were

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separately and continuously discharged from the centrifuge during operation.

Following separation of the RBCs, the RBCs were lysed to form a hemoglobin-containing solution. A substantial portion of the RBCs were mechanically lysed while discharging the RBCs from the centrifuge. The cell membranes of the RBCs ruptured upon impacting the wall of RBC phase discharge line at an angle to the flow of RBC phase out of the centrifuge, thereby releasing hemoglobin (Hb) from the RBCs into the RBC phase.

The lysed RBC phase then flowed through the RBC phase discharge line into a static mixer (Kenics $\frac{1}{2}$ inch with 6 elements, Chemineer, Inc.). Concurrent with the transfer of the RBC phase to the static mixer, an equal amount of WFI was also injected into the static mixer, wherein the WFI mixed with the RBC phase. The flow rates of the RBC phase and the WFI into the static mixer are each at about 0.25 lpm.

Mixing the RBC phase with WFI in the static mixer produced a lysed RBC colloid. The lysed RBC colloid was then transferred from the static mixer into a Sharples Super Centrifuge (Model # AS-16, Sharples Division of Alfa-Laval Separation, Inc.) which was suitable to separate the Hb from non-hemoglobin RBC components. The centrifuge was rotated at a rate sufficient to separate the lysed RBC colloid into a light Hb phase and a heavy phase. The light phase was composed of Hb and also contained non-hemoglobin components with a density approximately equal to or less than the density of Hb.

The Hb phase was continuously discharged from the centrifuge, through a 0.45 μm Millipore Pellicon Cassette, Cat # HVLP 000 C5 microfilter, and into a holding tank in preparation for Hb purification. Cell stroma were then returned with the retentate from the microfilter to the

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holding tank. During microfiltration, the temperature within the holding tank was maintained at 10 °C or less. To improve efficiency, when the fluid pressure at the microfilter inlet increased from an initial pressure of 5 about 10 psi to about 25 psi, microfiltration was complete. The Hb microfiltrate was then transferred from the microfilter into the microfiltrate tank.

Subsequently, the Hb microfiltrate was pumped through a 100,000 Millipore Cat # CDUF 050 H1 ultrafilter. A 10 substantial portion of the Hb and water, contained in the Hb microfiltrate, permeated the 100,000 Dalton ultrafilter to form a Hb ultrafiltrate, while larger cell debris, such as proteins with a molecular weight above about 100,000 Dalton, were retained and recirculated back into the 15 microfiltrate tank. Concurrently, WFI was continuously added to the microfiltrate tank as makeup for water lost in the ultrafiltrate. Generally, cell debris include all whole and fragmented cellular components with the exception of Hb, smaller cell proteins, electrolytes, coenzymes and 20 organic metabolic intermediates. Ultrafiltration continued until the concentration of Hb in the microfiltrate tank was less than 8 grams/liter (g/l). While ultrafiltering the Hb, the internal temperature of the microfiltrate tank was maintained at about 10 °C.

25 The Hb ultrafiltrate was transferred into an ultrafiltrate tank, wherein the Hb ultrafiltrate was then recirculated through a 30,000 Dalton Millipore Cat # CDUF 050 T1 ultrafilter to remove smaller cell components, such as electrolytes, coenzymes, metabolic intermediates and 30 proteins less than about 30,000 Daltons in molecular weight, and water from the Hb ultrafiltrate, thereby forming a concentrated Hb solution containing about 100 g Hb/l.

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The concentrated Hb solution was then directed from the ultrafiltrate tank onto the media contained in parallel chromatographic columns (2 feet long with an 8 inch inner diameter) to separate the Hb by high performance liquid chromatography. The chromatographic columns contained an anion exchange medium suitable to separate Hb from nonhemoglobin proteins. The anion exchange media was formed from silica gel. The silica gel was exposed to γ -glycidoxy propylsilane to form active epoxide groups and 10 then exposed to $C_3H_7(CH_3)_2NCl$ to form a quaternary ammonium anion exchange medium. This method of treating silica gel is described in the *Journal of Chromatography*, 120:321-333 (1976).

Each column was pre-treated by flushing the 15 chromatographic columns with a first buffer (Tris-acetate) which facilitated Hb binding. The pH of the buffer was about 9.0 ± 0.1 . Then 4.52 liters of the concentrated Hb solution were injected into each chromatographic column. After injecting the concentrated Hb solution, the 20 chromatographic columns were then washed by directing buffer solutions through the chromatographic columns to produce a stepped pH gradient of eluate from the columns. The temperature of each buffer during use was about 12.4 °C. The buffers were prefiltered through 10,000 Dalton 25 ultrafiltration membrane before injection onto the chromatographic columns.

In particular, a first buffer solution, 20 mM tris-hydroxymethyl aminomethane acetate (Tris-acetate) (pH about 8.4 to about 9.4), transported the concentrated Hb solution 30 in purified water (U.S.P.) into the media in the chromatographic columns to bind the Hb. A second buffer, having a pH of about 8.3, then adjusted the pH within chromatographic columns to elute contaminating non-hemoglobin components from the chromatographic columns,

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while retaining the Hb. Equilibration with the second buffer solution continued for about 30 minutes at a flow rate of approximately 3.56 lpm per column, or about 6.1 column volumes (11.7 void volumes). The eluate from the 5 second buffer was discarded to waste. A third buffer solution, 50 mM Tris-acetate (pH about 6.5 to about 7.5), then eluted the Hb from chromatographic columns as a purified hemoglobin product.

The Hb eluate was then directed through a sterile 10 0.22 μ Sartobran Cat # 5232507 G1PH filter to a tank wherein the Hb eluate was collected. The first 3-to-4% of the Hb eluate and the last 3-to-4% of the Hb eluate were directed to waste.

The Hb eluate was further used if the elute contained 15 less than 0.05 EU/ml of endotoxin and contained less than 3.3 nmoles/ml phospholipids. To sixty liters of ultrapure elute, which had a concentration of 100 g Hb/l, was added 9 1 of 1.0 M NaCl, 20 mM Tris (pH 8.9) buffer, thereby forming an Hb solution with an ionic strength of 160 mM, to 20 reduce the oxygen affinity of the Hb in the Hb solution. The Hb solution was then concentrated at 10 °C, by recirculating through the ultrafilter, specifically a 10,000 Dalton Millipore Helicon, Cat # CDUF050G1 filter, until the Hb concentration was 110 g/l.

25 The Hb solution was then deoxygenated, until the pO₂ of the Hb solution was reduced to the level where HbO₂ content was about 10%, by recirculating the Hb solution at 12 lpm, through a 0.05 μ m Hoechst-Celanese Corporation Cat # G-240/40) polypropylene microfilter phase transfer 30 membrane, to form a deoxygenated Hb solution (hereinafter "deoxy-Hb"). Concurrently, a 60 lpm flow of nitrogen gas was directed through the counter side of the phase transfer membrane. During deoxygenation, the temperature of the Hb solution was maintained between about 19 °C and about

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31 °C.

Also during deoxygenation, and subsequently throughout the process, the Hb was maintained in a low oxygen environment to minimize oxygen absorption by the Hb and to 5 maintain an oxygenated Hb (oxyhemoglobin or HbO₂) content of less than about 10% in the deoxy-Hb.

The deoxy-Hb 60 l, was then diafiltered through an ultrafilter with 180 l of a storage buffer, containing 0.2 wt % N-acetyl cysteine, 33 mM sodium phosphate buffer (pH 10 7.8) having a pO₂ of less than 50 torr, to form a oxidation-stabilized deoxy-Hb. Prior to mixing with the deoxy-Hb, the storage buffer was depyrogenated with a 10,000 Dalton Millipore Helicon, Cat # CDUF050G1 depyrogenating filter.

15 The storage buffer was continuously added at a rate approximately equivalent to the fluid loss across the ultrafilter. Diafiltration continued until the volume of fluid lost through diafiltration across the ultrafilter was about three times the initial volume of the deoxy-Hb. The 20 material may be stored at this point.

Prior to transferring the oxidation-stabilized deoxy-Hb into a polymerization apparatus, oxygen-depleted WFI was added to the polymerization reactor to purge the polymerization apparatus of oxygen to prevent oxygenation 25 of oxidation-stabilized deoxy-Hb. The amount of WFI added to the polymerization apparatus was that amount which would result in a Hb solution with a concentration of about 40 g Hb/l, when the oxidation-stabilized deoxy-Hb was added to the polymerization reactor. The WFI was then recirculated 30 throughout the polymerization apparatus, to deoxygenate the WFI by flow through a 0.05 µm polypropylene microfilter phase transfer membrane (Hoechst-Celanese Corporation Cat # 5PCM-108, 80 sq. ft.) against a counterflow of pressurized nitrogen. The flow rates of WFI and nitrogen gas, through

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the phase transfer membrane, were about 18 to 20 lpm and 40 to 60 lpm, respectively.

After the pO₂ of the WFI in polymerization apparatus was reduced to less than about 2 torr pO₂, the 5 polymerization reactor was blanketed with nitrogen by a flow of about 20 lpm of nitrogen into the head space of the polymerization reactor. The oxidation-stabilized deoxy-Hb was then transferred into the polymerization reactor.

The polymerization was conducted in a 12 mM phosphate 10 buffer with a pH of 7.8, having a chloride concentration less than or equal to about 35 mmolar which was produced by mixing the Hb solution with WFI.

The oxidation-stabilized deoxy-Hb and N-acetyl cysteine were subsequently slowly mixed with the cross- 15 linking agent glutaraldehyde, specifically 29.4 grams of glutaraldehyde for each kilogram of Hb over a five hour period, while heating at 42 °C and recirculating the Hb solution through a Kenics 1-1/2 inch static mixer with 6 elements (Chemineer, Inc.), to form a polymerized Hb 20 (poly(Hb)) solution.

Recirculating the oxidation-stabilized deoxy-Hb and the glutaraldehyde through the static mixer caused turbulent flow conditions with generally uniform mixing of the glutaraldehyde with the oxidation-stabilized deoxy-Hb, 25 thereby reducing the potential for forming pockets of deoxy-Hb containing high concentrations of glutaraldehyde. Generally uniform mixing of glutaraldehyde and deoxy-Hb reduced the formation of high molecular weight poly(Hb) (having a molecular weight above 500,000 Daltons) and also 30 permitted faster mixing of glutaraldehyde and deoxy-Hb during polymerization.

In addition, significant Hb intramolecular cross-linking resulted during Hb polymerization as an effect of

the presence of N-acetyl cysteine upon the polymerization of Hb.

After polymerization, the temperature of the poly(Hb) solution in the polymerization reactor was reduced to a 5 temperature between about 15°C to about 25°C.

The poly(Hb) solution was then concentrated by recirculating the poly(Hb) solution through the ultrafilter until the concentration of the poly(Hb) was increased to about 85 g/l. A suitable ultrafilter is a 30,000 Dalton 10 filter (e.g., Millipore Helicon, Cat # CDUF050LT).

Subsequently, the poly(Hb) solution was then mixed with 66.75 g sodium borohydride, to the poly(Hb) solution and then again recirculated through the static mixer. Specifically, for every nine liters of poly(Hb) solution, 15 one liter of 0.25 M sodium borohydride solution was added at a rate of 0.1 to 0.12 lpm.

Prior to adding the sodium borohydride to the poly(Hb) solution, the pH of the poly(Hb) solution was basified by adjusting pH to a pH of about 10 to preserve the sodium 20 borohydride and to prevent hydrogen gas formation. The pH of the poly(Hb) solution was adjusted by diafiltrating the poly(Hb) solution with approximately 215 l of depyrogenated, deoxygenated 12 mM sodium borate buffer, having a pH of about 10.4 to about 10.6. The poly(Hb) 25 solution was diafiltered by recirculating the poly(Hb) solution from the polymerization reactor through the 30 kD ultrafilter. The sodium borate buffer was added to the poly(Hb) solution at a rate approximately equivalent to the rate of fluid loss across the ultrafilter from 30 diafiltration. Diafiltration continued until the volume of fluid lost across the ultrafilter from diafiltration was about three times the initial volume of the poly(Hb) solution in the polymerization reactor.

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Following pH adjustment, sodium borohydride solution was added to the polymerization reactor to reduce imine bonds in the poly(Hb) solution to ketimine bonds and to form stable poly(Hb) in solution. During the sodium 5 borohydride addition, the poly(Hb) solution in the polymerization reactor was continuously recirculated through the static mixer and the 0.05 µm polypropylene microfilter phase transfer membrane to remove dissolved oxygen and hydrogen. Flow through a static mixer also 10 provided turbulent sodium borohydride flow conditions that rapidly and effectively mixed sodium borohydride with the poly(Hb) solution. The flow rates of poly(Hb) solution and nitrogen gas through the 0.05 µm phase transfer membrane were between about 2.0 to 4.0 lpm and about 12 to 18 lpm, 15 respectively. After completion of the sodium borohydride addition, reduction continued in the polymerization reactor while an agitator contained therein rotated at approximately 75 rotations per minute.

Approximately one hour after the sodium borohydride 20 addition, the stable poly(Hb) solution was recirculated from the polymerization reactor through the 30,000 Dalton ultrafilter until the stable poly(Hb) solution concentration was 110 g/l. Following concentration, the pH and electrolytes of the stable poly(Hb) solution were 25 restored to physiologic levels to form a stable polymerized Hb blood-substitute, by diafiltrating the stable poly(Hb) solution; through the 30,000 Dalton ultrafilter, with a filtered, deoxygenated; low pH buffer containing 27 mM sodium lactate, 12 mM NAC, 115 mM NaCl, 4 mM KCl, and 1.36 30 mM CaCl₂, in WFI, (pH 5.0). Diafiltration continued until the volume of fluid lost through diafiltration across the ultrafilter was about 6 times the pre-diafiltration volume of the concentrated Hb product.

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After the pH and electrolytes were restored to physiologic levels, the stable polymerized Hb blood-substitute was then diluted to a concentration of 5.0 g/dl by adding the filtered, deoxygenated low pH buffer to 5 polymerization reactor. The diluted blood-substitute was then diafiltered by recirculating from the polymerization reactor through the static mixer and a 100,000 Dalton purification filter against a filtered deoxygenated buffer containing 2.7 mM sodium lactate, 12 mM NAC, 115 mM NaCl, 4 10 mM KCl, and 1.36 mM CaCl₂ in WFI, (pH 7.8). Diafiltration continued until the blood-substitute contained less than or equal to about 10% modified tetrameric and unmodified tetrameric species by GPC when run under dissociating conditions.

15 The purification filter was run under conditions of low transmembrane pressure with a restricted permeate line. Following removal of substantial amounts of modified tetrameric Hb and unmodified tetrameric Hb, recirculation of the blood-substitute continued through the 30,000 Dalton 20 ultrafilter until the concentration of the blood-substitute was about 130 g/l.

The stable blood-substitute was then stored in a suitable container having a low oxygen environment and a low oxygen in-leakage.

25

EXAMPLE 2

POLYMERIZED HEMOGLOBIN ANALYSIS

The endotoxin concentration in the hemoglobin product is determined by the method "Kinetic/ Turbidimetric LAL 5000 Methodology" developed by Associates of Cape Cod, 30 Woods Hole, Massachusetts, J. Levin et al., J. Lab. Clin. Med., 75:903-911 (1970). Various methods were used to test for any traces of stroma for example, a precipitation

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assay, Immunoblotting, and enzyme-linked immunosorbent assay (ELISA) for a specific cell membrane protein or glycolipid known by those skilled in the art.

Particulate counting was determined by the method
5 "Particulate Matter in Injections: Large Volume Injections
for Single Dose Infusions", *U.S Pharmacopeia*, 22:1596,
1990.

To determine glutaraldehyde concentration, a
400 µl representative sample of the hemoglobin product was
10 derivatized with dinitrophenylhydrazine and then a 100 µl
aliquot of the derivative solution was injected onto a YMC
AQ-303 ODS column at 27 °C, at a rate of 1 ml/min., along
with a gradient. The gradient consisted of two mobile
phases, 0.1% trifluoroacetic acid (TFA) in water and 0.08%
15 TFA in acetonitrile. The gradient flow consisted of a
constant 60% 0.08% TFA in acetonitrile for 6.0 minutes, a
linear gradient to 85% 0.08% TFA in acetonitrile over 12
minutes, a linear gradient to 100% 0.08% TFA in
acetonitrile over 4 minutes hold at 100% 0.08% TFA in
20 acetonitrile for 2 minutes and re-equilibrate at 45% of
0.1% TFA in water. Ultraviolet detection was measured at
@360 nm.

To determine NAC concentration, an aliquot of
hemoglobin product was diluted 1:100 with degassed sodium
25 phosphate in water and 50 µl was injected onto a YMC AQ-303
ODS column with a gradient. The gradient buffers consisted
of a sodium phosphate in water solution and a mixture of
80% acetonitrile in water with 0.05% TFA. The gradient
flow consisted of 100% sodium phosphate in water for 15
30 minutes, then a linear gradient to 100% mixture of 80%
acetonitrile and 0.05% TFA over 5 minutes, with a hold for
5 minutes. The system was then re-equilibrated at 100%
sodium phosphate for 20 minutes.

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Phospholipid analysis was done by a method based on procedures contained in the following two papers:
Kolarovic et al., "A Comparison of Extraction Methods for the Isolation of Phospholipids from Biological Sources",
5 Anal. Biochem., 156:244-250, 1986 and Duck-Chong, C. G., "A Rapid Sensitive Method for Determining Phospholipid Phosphorus Involving Digestion With Magnesium Nitrate", Lipids, 14:492-497, 1979.

Osmolarity was determined by analysis on an Advanced
10 Cryomatic Osmometer, Model #3C2, Advanced Instruments, Inc., Needham, Massachusetts.

Total hemoglobin, methemoglobin and oxyhemoglobin concentrations were determined on a Co-Oximeter Model #482, from Instrumentation Laboratory, Lexington, Massachusetts.

15 Na⁺, K⁺, Cl⁻, Ca⁺⁺, pO₂ concentration was determined by a Novastat Profile 4, Nova Biomedical Corporation, Waltham, Massachusetts.

Oxygen binding constant, P₅₀ were determined by a
Hemox-Analyzer, TCS Corporation, Southhampton,
20 Pennsylvania.

Temperature and pH were determined by standard methods known by those skilled in the art.

Molecular weight (M.W.) was determined by conducting
gel permeation chromatography (GPC) on the hemoglobin
25 products under dissociating conditions. A representative sample of the hemoglobin product was analyzed for molecular weight distribution. The hemoglobin product was diluted to 4 mg/ml within a mobile phase of 50 mM Bis-Tris (pH 6.5), 750 mM MgCl₂, and 0.1 mM EDTA. This buffer serves to
30 dissociate Hb tetramer into dimers, that have not been cross-linked to other Hb dimers through intramolecular or intermolecular crosslinks, from the poly(Hb). The diluted sample was injected onto a TosohHaas G3000SW column. Flow

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rate was 0.5 ml/min. and ultraviolet detection was recorded at 280 nm.

The results of the above tests on veterinary (OXYGLOBIN™) and human (HEMOPURE™2) Hb blood-substitutes, 5 formed according to the method of invention, are summarized in Tables II and III, respectively.

Table II

PARAMETER		RESULTS
	pH (18-22°C)	physiologically acceptable pH
10	Endotoxin	< 0.5 EU/ml
	Sterility Test	Meets Test
	Phospholipids ^a	<3.3 nm/ml
15	Total Hemoglobin	12.0 - 14.0 g/dl
	Methemoglobin	<15%
15	Oxyhemoglobin	<10%
	Sodium, Na ⁺	145-160 mM
	Potassium, K ⁺	3.5-5.5 mM
	Chloride, Cl ⁻	105-120 mM
	Calcium, Ca ⁺⁺	0.5-1.5 mM
20	Boron	<10 ppm
	Osmolality	290-310 mOsm
	Glutaraldehyde	<3.5 µg/ml
	N-acetyl-L-cysteine	<0.2%
	M.W. >500,000	<15%
25	Unmodified Tetramer	<5%
	Particulate Content >10µ	<12/ml
	Particulate Content >25µ	<2/ml

^a measured in Hb before polymerization

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Table III

PARAMETER		RESULTS
pH (18-22°C)		Physiologically acceptable pH
	Endotoxin	< 0.5 EU/ml
5	Sterility Test	Meets Test
	Phospholipids ^a	<3.3 nm/ml
	Total Hemoglobin	12.0 - 14.0 g/dl
	Methemoglobin	<15%
	Oxyhemoglobin	<10%
10	Sodium, Na ⁺	145-160 mM
	Potassium, K ⁺	3.5-5.5 mM
	Chloride, Cl ⁻	105-120 mM
	Calcium, Ca ⁺⁺	0.5-1.5 mM
	Boron	<10 ppm
15	Osmolality	290-310 mOsm
	Glutaraldehyde	<3.5 µg/ml
	N-acetyl-L-cysteine	≤0.2%
	M.W. >500,000	≤15%
	M.W. ≤ 65,000	<10%
20	M.W. <32,000	<5%
	Particulate Content >10µ	<12/ml
	Particulate Content >25µ	<2/ml

^a measured in Hb before polymerization

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EXAMPLE 3

DETERMINATION OF IN VIVO ONCOTIC EFFECTS IN CANINES

The purpose of this study was to determine the in vivo oncotic effects, specifically the volume of water drawn 5 into the intravascular space per gram of hemoglobin administered, of veterinary (OXYGLOBIN™) Hb blood-substitute in splenectomized beagle dogs by measuring the expansion of plasma volume following a toploading dose. In addition, a comparable dose of (RHEOMACRODEX™-Saline), 10 manufactured by Pharmacia, which is 10% Dextran 40 and 0.9% saline, was also determined.

Two dogs were entered into this study after a routine health screening and an acclimatization period of at least four weeks. The dogs were splenectomized at least 3 days 15 before treatment. They were pre-anesthetized, with a combination of atropine and meperidine HCl, and anesthetized via inhalation of isofluorane. Lactated Ringer's solution was infused at 10-20 ml/kg/hr during the surgical procedure.

20 The dogs received the Hb blood-substitute (40 ml/kg) at 20 ml/kg/hr via a disposable cephalic catheter. Hematocrit was measured pre-dosing and at 1/4, 1/2, 1, 2, 3, 4 hours post-dosing or longer until the nadir of the hematocrit was established.

25 The dogs were splenectomized to ensure a constant plasma volume and RBC mass to allow accurate measurement of the change in plasma volume following dosing.

Calculation of the change in plasma volume was made using the following equation:

$$\Delta \% PV = \left\{ \frac{Hct_1(1-Hct_2)}{Hct_2(1-Hct_1)} - 1 \right\} 100$$

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where PV is the plasma volume, Hct₁ is the initial hematocrit, and Hct₂ is the final hematocrit. This calculation was based on the change in hematocrit, assuming that the number of RBC's within the circulating blood 5 volume and mean corpuscular volume remained constant.

As shown in Table IV, the nadir of the hematocrit occurred two hours post-dosing in both dogs. The mean corpuscular volume (MCV) remained stable throughout the study.

10

Table IV

Time (Hour)	Hematocrit (%)		MCV (fL)	
	Dog 3503C	Dog 14 Male	Dog 3503C	Dog 14 Male
15	0	46	67.6	67.2
	1/4	41	68.1	67.7
	1/2	37	67.5	67.2
	1	35	68.6	67.9
	2	31	68.1	67.1
	3	33	66.8	66.1
	4	32	66.3	65.4

The volume of fluid drawn intravascularly post dosing 20 was 6 ml/g hemoglobin and 9 ml/g hemoglobin for dogs 3503C and 14 male, respectively. The dose of synthetic colloid solution (Rheomacrodex®-Saline) was calculated based on a dose that causes a similar oncotic effect. Rheomacrodex draws approximately 22 ml fluid from the interstitium per 25 gram administered intravenously.

The calculated comparable dose of Rheomacrodex was 14 ml/kg and 7 ml/kg for 30 ml/kg and 15 ml/kg Hb blood-substitute, respectively.

The volume of fluid drawn intravascularly by 30 (Oxyglobin™) Hb blood-substitute was 8 ml H₂O/gram

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hemoglobin. Since the volume of the dose was 30 ml/kg, and the concentration of hemoglobin in the dose was 13 g/dl, the total amount of hemoglobin per dose was 3.9 g/kg and the total volume of fluid drawn into the intravascular space/dose by the Hb blood-substitute was 31.2 ml

The synthetic colloid solution draws in about 22 ml of water/gram of Dextran. The total amount of Dextran in the colloid solution per comparable dose of Hb blood-substitute is 1.4 g. Thus, the total volume of fluid drawn into intravascular space/comparable dose of colloid solution is 14 ml.

EXAMPLE 4
CANINE DOSE RESPONSE STUDY

This study was conducted to determine the drug effect and dose response of veterinary (OXYGLOBIN™) Hb blood-substitute of this invention, as compared to a synthetic colloid solution, of (RHEOMACRODEX™-Saline, Pharmacia) which is 10% Dextran 40 and 0.9% saline, with respect to arterial oxygen content relative to canine red blood cell hemoglobin and oxygen delivery in splenectomized beagle dogs 60 minutes and 24 hours following acute normovolemic hemodilution.

Acute normovolemic hemodilution is an experimental model that mimics a clinical condition of anemia due to surgical blood loss. Severe anemia (Hct = 9%, Hb = 3 g/dl) was produced by this method to cause an absolute requirement of oxygen carrying support. Oxygen delivery and oxygen content decreased precipitously with the massive bleeding.

In developing the normovolemic hemodilution model, it was found that treatment to restore oxygen delivery either by volume expansion alone, as was done for the control

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dogs, or by volume expansion in conjunction with an increase in the arterial oxygen content, as occurred for the dogs treated with hemoglobin solution, had to occur within approximately 10 minutes of reaching a hematocrit of 5 9% to avoid irreversible decreases in blood pressure and cardiac output which then resulted in death.

Two of 12 control dogs in this study died during or following dosing even though their vascular volume was expanded with Dextran 40 solution within 5 minutes of 10 reaching the targeted hematocrit. The death of these dogs is a reflection of the severity of the experimental model which in turn portrays the clinical condition of severe acute blood loss.

Thirty dogs were entered into this study after a 15 routine health screening and acclimatization period of at least four weeks. Treatment was staggered using three replicates of dogs (A, B and C), each replicate containing one dog/sex/group. Dogs were randomly assigned to the 5 groups (6 dogs/group of 3 males and 3 females) 32 days 20 before the first day of treatment. Dogs were assigned to their respective groups by block randomization based on body weight using a method which ensured equal distribution among groups. Males and females were randomized separately. Any dog with unacceptable pretreatment data, 25 such as abnormal clinical signs or clinical pathology data, was replaced by a spare dog maintained under the same environmental conditions.

The test/control articles were administered by a single intravenous infusion. The rate of infusion was 30 controlled by an infusion pump. The actual volume infused per hour depended upon the most recent body weight of each of the dogs.

The highest dose of hemoglobin solution was based upon the safe upper limit of acute cardiovascular effects due to

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volume expansion in normovolemic dogs. The mid-range dose was chosen to define the shape of the dose response curve. The lowest dose was based on the lower limit of clinically relevant dosing as defined by volume and hemodynamic
5 effects in the dog.

Each dog was splenectomized at least 7 days before treatment to avoid effects on the experimental model of an increased circulatory RBC mass due to splenic contraction. On the day of treatment with hemoglobin solution, each dog
10 was anesthetized by inhalation of isoflurane and mechanically ventilated using room air with a tidal volume of 20-25 ml/Kg. The rate of ventilation was adjusted during the procedure to maintain arterial pCO₂ at approximately 40 mmHg. The end-expired concentration of
15 isoflurane was measured and controlled to provide a valid comparison of anesthetic plane from dog to dog. The dogs were instrumented for monitoring of hemodynamic function and oxygen transport parameters. Placement of a flow-directed catheter in the pulmonary artery was confirmed by
20 analysis of pressures and pressure tracings. A dual-lumen catheter, with thermodilution cardiac output capability, was placed in the femoral artery to provide an arterial line for blood pressure monitoring and blood withdrawal. A catheter was placed in the cephalic vein, or other vein if
25 required, for volume replacement and test/control article administration.

Each dog received an intramuscular injection of antibiotics once daily (Procaine penicillin G) prophylactically for one day prior to surgery, on the day
30 of surgery and for 3 days following the splenectomy. V-Sporin, a topical antibiotic (Polymyxin B, Bacitracin, Neomycin) was applied to the surgical site once daily, as needed.

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Following instrumentation, hemodynamic stabilization to reach a pCO₂ of approximately 40 mm Hg and collection of baseline measurements were performed. A model of acute normovolemic hemodilution was then produced by bleeding the 5 dogs and simultaneously replacing approximately 1.6 to 2.3 times the volumes withdrawn with Lactated Ringer's Solution to maintain isovolemic status. Isovolemic status was achieved by maintaining pulmonary artery wedge pressure at approximately baseline values. The blood withdrawal/volume 10 replacement took approximately 45 to 90 minutes until the hemoglobin concentration was approximately 30 g/l (3.0 g/dl). Lactated Ringer's Solution was infused rapidly using a gravity intravenous set and a pressure cuff around the infusion bag. If the arterial systolic blood pressure 15 was ≤ 50 mmHg for more than 5 minutes following the induction of acute anemia and prior to the start of dosing, the dog was rejected and replaced by a spare dog maintained under the same environmental conditions.

Doses of colloid control and hemoglobin solution were 20 administered as stated in Table V. Hemodynamic measurements were performed pre-bleed, pre-dose, immediately following dosing, and at 60 minutes and 24 hours following dosing. After the 60 minute measurement, the dog recovered from anesthesia and was instrumented 25 again for hemodynamic measurements, performed at 24 hours following dosing.

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Table V

Group	Test Article	Dose Volume ml/Kg	Dose Rate ml/Kg/h	Animals/Group	
				Males	Females
5	1 Colloid control (mid dose)	14	20	3	3
	2 Colloid control (low dose)	7	20	3	3
	3 Hb blood-substitute (low dose)	15	20	3	3
	4 Hb blood-substitute (mid dose)	30	20	3	3
	5 Hb blood-substitute (high dose)	45	20	3	3

All hemodynamic parameters were statistically analyzed by either analysis of variance (ANOVA) or analysis of covariance (ANCOVA) with either the pre-bleeding or pre-dosing value as the covariate. Specific linear contrasts were constructed to test for the effects of volume of the solution administered, the effect of Hb blood-substitute (drug effect), and the dose response of the Hb blood-substitute (dose effect). These tests were performed only for parameters for which the difference among experimental groups was statistically significant at the 0.05 level. Comparisons of specified variables at selected time points were performed by paired t-tests in each group.

Arterial oxygen content was one criterion of efficacy in this study. Arterial oxygen content is a measure of the oxygen carrying capacity of cellular and plasma hemoglobin and dissolved oxygen in the plasma. In the absence of plasma hemoglobin, arterial oxygen content is calculated from the amount of oxygen carried by saturated cellular hemoglobin and the partial pressure of inspired oxygen.

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Because plasma hemoglobin was expected to contribute significantly to oxygen content in this study, oxygen content was measured directly using a LexO2Con-K instrument (Chestnut Hill, MA). Oxygen enriched air was not

5 administered during the experiment because it was unnecessary and to avoid the confounding effects of an increased inspired oxygen concentration on the measurement of arterial oxygen content.

Mean arterial and venous oxygen contents decreased
10 approximately four and eight times, respectively in all groups following induction of anemia. Arterial oxygen content increased significantly 60 minutes following dosing compared to pre-dosing values in all Hb blood-substitute treated groups and remained significantly increased at 24
15 hours following dosing in the mid and high dose groups. Arterial or venous oxygen content did not change following dosing in either control group.

As shown in Figure 2, arterial oxygen content was significantly increased in Hb blood-substitute treated
20 groups compared to control groups at 60 minutes and 24 hours following dosing. A linear dose response was seen at 60 minutes and 24 hours following dosing. A significant volume effect was detected for arterial oxygen content 60 minutes following dosing.

25 Venous oxygen content also significantly increased in Hb blood-substitute treated groups compared to controls at 60 minutes and 24 hours following dosing. The increase showed a linear dose response at 60 minutes following dosing but not at 24 hours.

30 The dose effect observed for Hb blood-substitute treated groups in arterial-venous (A-V) oxygen content difference at 60 minutes following dosing was attributed to significant volume effects based on the absence of a drug effect and similar observations of volume effects in

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control groups at 60 minutes following dosing. Hb blood-substitute treated groups showed a significant increase in A-V oxygen difference at 24 hours compared to colloid controls, with a significant linear dose response. The A-V
5 difference must be interpreted in view of the cardiac output. At 24 hours following dosing, the A-V difference in the control groups was significantly lower than that of the Hb blood-substitute treated groups. One possible explanation for this difference is that the control group
10 dogs had to rely on a higher cardiac output to meet the oxygen consumption needs of peripheral tissues. The Hb blood-substitute treated groups maintained a large enough A-V difference at 24 hours following dosing to meet peripheral tissue needs without cause for an increased
15 cardiac output.

In addition to arterial oxygen content, total arterial oxygen content normalized relative to the contribution of canine RBC hemoglobin ($\text{CaO}_2/\text{g RBC Hb}$) was examined in this study. This comparison was made to demonstrate differences
20 in arterial oxygen content among dosing groups since the RBC hemoglobin was constant in all groups. The potential correlation of plasma or total hemoglobin concentration and arterial oxygen content would provide a useful clinical measure of efficacy. As shown in Figure 3, at 60 minutes
25 and 24 hours following dosing, all Hb blood-substitute treated groups (except the low dose group at 24 hours) showed a significant increase in $\text{CaO}_2/\text{g RBC hemoglobin}$ to pre-dosing values. Arterial oxygen content relative to that contributed by RBC hemoglobin did not differ
30 significantly in the colloid controls between pre-dose and 60 minutes or 24 hours following dosing.

Total arterial oxygen content relative to that contributed by red blood cell hemoglobin significantly increased in Hb blood-substitute treated groups compared to

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colloid controls at 60 minutes following dosing with a significant linear dose response. A significant dose effect also occurred at 24 hours following dosing with a significant linear dose response, but the drug effect was
5 not quite significant ($P<0.06$).

Oxygen delivery was another criterion of efficacy. Oxygen delivery is calculated based on arterial oxygen content and cardiac output. Therefore, oxygen delivery is affected by all the physiologic factors which influence
10 cardiac output. The control chosen for this study was the synthetic colloid (RHEOMACRODEX™-Saline, Pharmacia) which is 10% Dextran 40 and 0.9% saline, as it expands intravascular volume and is not known to carry oxygen. The control provided a comparison of equivalent volume
15 expansion to the colloidal properties of the hemoglobin in Hb blood-substitute.

Because each dose of Hb blood-substitute was expected to demonstrate a distinct volume effect, two doses of dextran solution were used as controls for the volume
20 effect so the data would reflect only the drug effect of different doses. This comparison was made for the low and mid doses. The doses of colloid controls were selected based on those doses of Dextran 40 which provided an equivalent comparison of the *in vivo* oncotic effects of the
25 low and mid-dose test articles, as determined from the results of Example 2.

The volume effect was defined statistically using the difference in means between the colloid mid dose (14 ml/kg) and the colloid low dose (7 ml/kg). The drug effect was
30 determined by comparing each Hb blood-substitute treated group to its corresponding colloid control. A linear dose response was established when a statistically significant difference was seen between the low and high dose Hb blood-substitute treated groups.

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Oxygen delivery was calculated according to the equation: $\text{DO}_2 = \text{CO} \times \text{CaO}_2 \times 10/\text{kg}$ which CO is the cardiac output and CaO₂ is the arterial oxygen content. As expected, following induction of anemia in all treatment groups, a two to three fold mean decrease in DO₂ occurred in all groups. The oxygen content decreased sufficiently that the maintenance of baseline oxygen consumption had to result from an increase in cardiac output and increased extraction of oxygen, resulting in a lower venous oxygen content. As shown in Figure 4, oxygen delivery increased approximately 30% in the low dose Hb blood-substitute treated group and greater than 100% in the mid and high Hb blood-substitute treated groups at 60 minutes following dosing compared to pre-dosing values. The difference was significant for all three dosing groups ($p<0.05$). The control groups showed no significant differences over this time. At 60 minutes following dosing, DO₂ differed significantly among all groups with significant drug and dose effects with a linear dose response. At 24 hours, no difference in oxygen delivery was noted among groups. The improvement in oxygen delivery at 60 minutes following dosing for all Hb blood-substitute treated groups, as compared to their corresponding colloid controls, was due primarily to a dose related increase in arterial oxygen content in addition to a modest increase in cardiac output.

Oxygen consumption was calculated according to the equation: $\text{VO}_2 = \text{CO} \times \text{CaO}_2 \times 10 \text{ kg}$. A two to three fold mean decrease in DO₂ occurred in all groups following the induction of anemia. No statistically significant differences were noted among Hb blood-substitute treated or control groups or within a group when comparing pre-dosing to post-dosing values.

The Oxygen Extraction Ratio (VO₂/DO₂) for all groups showed an approximately three fold increase following

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induction of anemia. Oxygen extraction ratios were significantly decreased in a dose dependent manner in all Hb blood-substitute treated groups at 60 minutes following dosing compared to control groups. No significant 5 differences were noted between Hb blood-substitute treated and control groups at 24 hours following dosing.

Mean cardiac output increased between 10% and 39% in all groups following induction of anemia. Cardiac output was significantly increased at 24 hours following dosing 10 compared to pre bleeding values in the colloid control groups but not in the Hb blood-substitute treated groups. A significant volume effect which contributed to significant differences in cardiac output between colloid low and mid dose groups was evident at 60 minutes post- 15 dosing. The increase in cardiac output was likely related to an increased stroke volume due to expansion of the intravascular volume following dosing or increased sympathetic tone due to the stress of severe anemia. A significant dose response between Hb blood-substitute low 20 and high dose groups was apparent at 60 minutes, but not at 24 hours following dosing. No difference in cardiac output between Hb blood-substitute treated and colloid control groups was seen at 60 minutes or 24 hours following dosing.

Pulmonary artery wedge pressure (PAWP) did not change 25 significantly during the induction of anemia. PAWP decreased significantly in the low dose colloid group and remained unchanged in the mid dose colloid group 60 minutes following dosing compared to pre dosing values. The PAWP in the mid and high dose Hb blood-substitute treated groups 30 increased significantly in a linear dose response compared to pre-dosing values at 60 minutes following dosing. The increased PAWP reflected a dose dependent increase in intravascular volume at 60 minutes following dosing. No significant drug effect was detected between Hb blood-

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substitute treated and control groups at 60 minutes or 24 hours following dosing. A significant volume effect was detected in the colloid control groups at 60 minutes following dosing.

5 Systolic, diastolic and mean arterial blood pressure decreased significantly in all groups following induction of anemia, then increased significantly immediately following dosing. The decrease in systolic arterial blood pressure after the induction of anemia was likely related
10 to a decrease in peripheral vascular resistance due to decreased blood viscosity, a consequence of anemia. At 60 minutes following dosing, the systolic, diastolic, and mean arterial blood pressures of both colloid control groups did not differ significantly from pre-dosing values. The
15 systolic, diastolic, and mean pressures of the low dose colloid control increased significantly compared to pre-dosing values at 24 hours following dosing. In contrast, the increase in systolic, diastolic, and mean pressures was statistically significant in all Hb blood-substitute
20 treated groups at 60 minutes and 24 hours following dosing compared to pre-dosing values. The systolic, diastolic and mean blood pressures of Hb blood-substitute treated groups were significantly higher than corresponding colloid control groups at 60 minutes following dosing, but not at
25 24 hours.

Significant increases in systolic, diastolic and mean pulmonary arterial pressures were observed in the mid and high dose Hb blood-substitute treated groups 60 minutes post dosing compared to pre dosing values. The increases
30 persisted at 24 hours post-dosing in the mid Hb blood-substitute treated group for pulmonary diastolic arterial pressure. Additionally the low-dose colloid group showed a statistically significant increase at 24 hours post-dosing compared to pre-dosing values for mean pulmonary artery

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pressure. This increase was considered clinically significant. The increases in systemic arterial systolic and diastolic blood pressure 60 minutes following dosing of Hb blood-substitute, compared to pre-dosing values, were a 5 direct drug effect of the Hb blood-substitute. The diastolic pressure remained unchanged in the colloid control groups which was probably a result of a decreased peripheral vascular resistance.

No significant differences were found between Hb 10 blood-substitute treated and control groups for pulmonary systolic arterial pressure at either 60 minutes or 24 hours post-dose. In contrast, pulmonary diastolic and mean arterial pressures were significantly different with regard to volume, drug, and dose effects at 60 minutes post 15 dosing, but not at 24 hours.

Total hemoglobin decreased approximately four times or greater with bleeding. Hb blood-substitute treated groups showed a dose dependent increase in total hemoglobin compared to corresponding colloid control groups at 60 20 minutes and 24 hours following dosing.

Plasma hemoglobin concentrations significantly increased in a dose dependent manner in Hb blood-substitute treated groups compared to corresponding colloid control groups at 60 minutes and 24 hours following dosing. The 25 increases in plasma and total hemoglobin concentrations following dosing in all Hb blood-substitute treated groups, as compared to their corresponding colloid controls, were attributable to the hemoglobin content of Hb blood-substitute. The dose dependent significant increase 30 persisted for 24 hours, correlating with the persistent increase in arterial oxygen content.

In summary, the response to treatment with the Hb blood-substitute was linear, i.e., at 60 minutes following dosing, the higher the dose of Hb blood-substitute the

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greater the improvement in oxygen delivery and hemodynamics compared to corresponding colloid controls. Sustained arterial oxygen content and normal clinical signs, while breathing room air, support a beneficial biological effect

5 of Hb blood-substitute lasting 24 hours in the 30 ml/kg and 45 ml/kg dose Hb blood-substitute treated groups. The clearance of Hb blood-substitute likely accounts for the changes seen in oxygen delivery and hemodynamic effects at 24 hours following dosing. In conclusion, results from

10 this study support selection of a dose ranging from 30 to 45 ml/kg. Both of these dosing groups showed statistically significant differences from corresponding colloid control groups in the parameters of efficacy and the dose response was linear.

15 The clinical rationale of this dosing range is based on the fact that a severely anemic dog (e.g., hematocrit <15% with marked clinical signs) would benefit from a higher dose as demonstrated by the linear dose response of improved arterial oxygen content and oxygen delivery.

20 However, a more conservative dose would be indicated for a dog which may be predisposed to intravascular volume overload. The dose dependent transient increase in pulmonary artery wedge pressure and pulmonary arterial pressures seen 60 minutes following dosing in Hb blood-

25 substitute treated groups would limit the use of a higher dose in this population of dogs. Therefore a dosing range of 30-45 ml/kg would be effective in a broad population of dogs in which the degree of anemia and intravascular volume status are defined.

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EXAMPLE 5
HUMAN DOSE RESPONSE STUDY

This study was conducted to evaluate the safety and tolerance of increasing rates of intravenous administration 5 of Hb blood-substitute (hereinafter HBOL) upon hemodynamic, neuroendocrine and hematologic parameters in humans. The test subjects were normal healthy adult males (70-90 kg) between the ages of 18-45 years. During the study, the test subjects were on controlled isocaloric diets of 55% 10 carbohydrates, 30% fat (polyunsaturated to saturated fat ratio of 2:1), 15% protein and 150 mEq of sodium per day. Fluid intake was at least 3000 mls/day with caffeine containing beverages avoided. Also concomitant use of medication was avoided. Further no alcohol or tobacco were 15 used by the test subjects during the study.

The 12 subjects studied, were divided into three test groups. In each test group, three subjects received HBOL and one served as a control, receiving Ringer's lactate. Each test group had different rates of HBOL infusion. The 20 study was conducted as a single-blind, rate escalation study over a thirty day interval.

On Day 1 of the study, during the inpatient phase, each subject had a small gauge arterial catheter inserted in the radial artery of the non-dominant hand. The 25 insertion location was cleansed with an antiseptic solution (alcohol and/or iodine) and then a small amount of 1%-2% Lidocaine anesthetic solution was subcutaneously injected over the site of the radial artery. The arterial catheter was inserted to monitor blood pressure and to facilitate 30 blood gases evaluations. One to two hours later, all subjects had one large-bore intravenous catheter (16-gauge needle in antecubital fossa) placed in a vein in one arm. Each subject then had a phlebotomy of 750 ml (1.5 units) of

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whole blood drawn in less than 15 minutes, which was then followed with isovolemic hemodilution by the infusion of 2250 ml of Ringer's lactate over a 2 hour period.

Forty-five grams (346 ml) of HBOL were then

5 intravenously infused using sterile technique, in series through a standard 80 micrometer blood filter, a 5 micrometer filter, and the large-bore intravenous catheter in the arm vein, into each subject in the test groups 1, 2 and 3 at the rates of 0.5 gm/minute, 0.75 gm/minute and 1.0
10 gm/minute, respectively.

Simultaneously, each subject had invasive monitoring by radial artery catheter, serial pulmonary function tests, cardiac function evaluation and multiple hematology, chemistry and urinalysis laboratory tests which were

15 routinely and frequently performed over the first 28 hours after commencing HBOL infusion.

Subsequently, in the outpatient phase (Days 2-29), laboratory studies, vital signs, ECGs and medical events were taken daily for the first four days post-discharge and
20 then on a weekly basis for a month.

Hemodynamics were remarkable for generally higher values for systolic, diastolic and mean arterial pressure in the HBOL-treated groups (after infusion) than controls during Day 1. Although there was marked variability in the
25 blood pressure data commensurate with patient activity (e.g., during meals or when using the bathroom) and diurnal rhythm, HBOL-treated subjects generally had values for the systolic blood pressure (about 5-15 mm Hg), diastolic blood pressure (about 5-10 mm Hg) and mean arterial pressure
30 (about 10 mm Hg) greater than controls only during the course of Day 1. Values tended to reach peak effects between Hours 8-12 with return to baseline during sleep and upon removal of the arterial catheter. Pulse was generally about 10 beats lower in all HBOL-treated groups compared to

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controls during Day 1. The nadir of pulse decline was seen within the first 15 minutes of the infusion. Values were similar in all test groups after hour 24.

Cardiac index declined about 1-2 l/min/m² during the 5 first hour of infusion remained up to 1 l/min/m² lower than controls through hour 4, and then it returned to baseline by hour 4. Cardiac index also increased during times of patient activity (as above).

Total peripheral resistance paralleled blood pressure 10 changes, however, values returned to baseline within two hours. The transient increase in systemic blood pressure with an increase in total peripheral resistance and decrease in cardiac index is not unexpected. It is important to note that there was no difference in the rate 15 of administration and the magnitude of these hemodynamic responses and that no intervention was indicated.

The pulmonary function tests (including multiple determinations of spirometry and lung volumes) and arterial blood gas measurements were unremarkable. What was 20 noteworthy was the enhanced diffusion capacity that was seen in the HBOL-treated groups. The 10-15% increase in diffusion capacity was statistically significant compared to a 10% decrease in the controls for up to 24 hours. These findings are particularly important because of the 25 magnitude of phlebotomy and hemodilution that all of the groups underwent.

In hematological studies, other than the expected, transient decline in hemoglobin, hematocrit, red cell count and serum proteins with the phlebotomy and hemodilution 30 procedures, the hematology and serum chemistry laboratory tests were unremarkable. Exceptions were serum iron and ferritin which showed peak values by Hours 6 and 48, respectively, after HBOL was given.

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The serum chemistry measurements were unremarkable, with the exception of one subject (#10) who had transient increases in serum transaminases and lipase. It is important to note that this subject did not have any 5 clinically significant concomitant medical events (e.g., dysphagia or abdominal pain) commensurate with the time of the elevation of these enzymes. The exact etiology of these laboratory abnormalities is unclear, but previous studies suggest that transient subclinical spasm of the 10 sphincter of Oddi or other portions of the hepatobiliary and pancreatic ductal systems may be involved. It is important to note that these changes were transient (and unaccompanied by abdominal discomfort) and without apparent sequelae. No significant change was noted in Subject #10's 15 post-dose ultrasound of the gall bladder.

Urinalysis was unremarkable throughout the study. There was no detectable urinary hemoglobin in the subjects during the study. In addition, creatinine clearance was slightly higher, as expected, during the hemodilution 20 period), urinary adenosine deaminase binding protein, electrolytes (sodium, potassium, chloride), iron, microalbumin, NAG (N-acetyl-beta-glucosaminidase) and urinary urea nitrogen were unremarkable.

No apparent changes in the majority of the 25 pharmacokinetic parameters were observed as a function of administration rate. Sequential blood specimens and cumulative urine specimens were collected prior to and following initiation of infusion of HBOL for size exclusion (gel filtration) chromatographic (SEC) analysis of total 30 hemoglobin and apparent molecular weight fractions of hemoglobin. Only sporadic plasma dimer fraction concentrations were observed precluding any pharmacokinetic analysis. The only statistically significant differences ($p < 0.05$) were observed in the tetramer volume of

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distribution (decreases with increases in rate), tetramer maximum concentration achieved (increases with increases in rate) and the time of the tetramer maximum concentration occurrence (decreases with increases in rate).

5 The observed medical events were consistent with expected findings related to phlebotomy (e.g., vasovagal episode), multiple pulmonary function tests (aerophagia, eructation or abdominal "gas"), arterial line insertion (e.g., pain or tingling over the site), or abdominal
10 discomfort (e.g., associated with the ingestion of the iron supplement). Although there seemed to be a background of nonspecific, transient abdominal "gas." there were no cases of overt abdominal pain or dysphagia. In addition there was no correlation of these symptoms with any alterations
15 in serum transaminases or lipase.

In summary, HBOL was well tolerated. Although there were small transient increases in blood pressure and total peripheral resistance with commensurate decline in cardiac index during the first two hours of the infusion, the
20 hemodynamics were unremarkable. The increase in diffusion capacity was significantly higher in the HBOL-treated groups than controls during the first 24 hours.

EXAMPLE 6
EFFECTS OF HBOL ON HUMANS
IN GRADED BICYCLE EXERCISE TESTING

This study was conducted to evaluate the exercise capacity of subjects given autologous transfusion of HBOL. Specific endpoints included pulmonary function (e.g., diffusion capacity and lactic acid levels and pO₂),
30 hemodynamics (e.g., heart rate, cardiac index and blood pressure) and exercise tolerance (e.g., duration, workload and anaerobic threshold). The subjects were six normal

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healthy male humans, ages 18-45 years. One subject was replaced in the study due to failure to obtain the volume of phlebotomy in less than 15 minutes. The study was conducted as a randomized, single-blind, two-way crossover
5 study.

All subjects had phlebotomy of 750 ml followed by Ringer's Lactate [3:1] and either an autologous transfusion (ATX) or 45 gms of HBOL. The ATX or HBOL was given at 0.5 gm/min for 90 minutes. Bicycle exercise stress
10 tests were done on the day prior to phlebotomy and approximately 45 minutes after the infusion of ATX or HBOL. The same procedures were repeated one week later and subjects were crossed over to the opposite treatment.

On the day of dosing (Days 1 and 8), all subjects had
15 insertion of an arterial line in one radial artery, attachment to cardia telemetry and impedance cardiography and then phlebotomy (PBX) of 750 ml of whole blood (< 15 minutes). This was followed by an infusion of 2250 ml of Ringer's lactate (RL) over two hours (the isovolemic
20 hemodilution phase). Subjects then received either HBOL (45 gms [about 346 - 360 ml] at a rate of 0.5 gm/min over 90 minutes) or an ATX (110 -120 gms of hemoglobin [about 750 ml] at the same rate and duration as the HBOL). The BEST was done about 45 minutes after the end of either
25 infusion. Serial measurements of arterial blood gases, hematology, chemistry and urine tests were made intensively during the 24 hour period on Days 1 and 8. Serial follow-up was done on an outpatient basis between the dosing and for one month after all dosing was complete.

30 Subjects were able to exercise to similar levels during HBOL and ATX periods. The oxygen uptake (VO_2) and carbon dioxide production (VCO_2) at anaerobic threshold were nearly identical. The actual workload in METS, watts, pulse (as a % of maximum pulse), time to anaerobic

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threshold, tidal volume (VT) and minute ventilation (VE) were also similar. Arterial blood gas values were similar during the HBOL and ATX periods. The small reductions in pH and bicarbonate with increase in lactic acid is
5 consistent with expected findings at anaerobic threshold. The results of these bicycle tests showed that exercise capacity (defined as time and workload to reach anaerobic threshold) was similar at baseline and after infusions of either autologous transfusion or HBOL. Specifically,
10 hemodynamics were remarkable for slightly higher values (~ 5 mmHg) during the HBOL period for systolic, diastolic and mean arterial pressure. Commensurate with the increase in blood pressure was an increase in total peripheral resistance, generally within the first 4 hours. Cardiac
15 index declined during the HBOL period (~ 0.5 l/min/M²). Pulse was about 5-10 beats lower during the HBOL than the ATX period. These findings have been observed in the HBOL studies and have been of little clinical concern.

Pulmonary function tests were unremarkable except for
20 a 14% increase above baseline in diffusion capacity after the ATX and HBOL infusions. Subjects were able to achieve similar exercise capacity during HBOL and ATX periods. Arterial blood gas measurements during peak exercise (anaerobic threshold) were similar in both periods, but
25 arterial pO₂ tended to be higher during the HBOL period. Plasma lactic acid levels were lower during the HBOL than ATX period. Resting metabolic art measurements indicated that oxygen consumption, carbon dioxide production and metabolic energy expenditure were greater during the HBOL
30 than ATX period. The comparison as mentioned above is roughly one gram of HBOL to 3 grams of ATX. The diffusion capacity coupled with the observations about VO₂ and VCO₂ indicate that more oxygen is being delivered to the tissue level per gram of HBOL than ATX. It is commonly held that

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the diffusion capacity varies directly with the hemoglobin level, however, there is a suggestion that 1 gram of plasma hemoglobin may increase diffusion capacity as much as 3 gms of RBC hemoglobin.

5 Laboratory studies were notable for small, but transient increases in ALT, AST, 5'-nucleotidase, lipase and creatine kinase during the HBOL period. There were no abnormal urinary finding.

Hematological studies were consistent with those in
10 Example 5.

The observed medical events were consistent with expected findings related to the phlebotomy (e.g., vasovagal episode), multiple pulmonary function tests (eructation or abdominal "gas"), arterial line insertion
15 (e.g., pain or tingling over the insertion site) or numerous everyday complaints that one might observe in normal subjects over the course of a month (e.g., headache, upper respiratory tract infection or cold). The one subject (Subject #105) that had abdominal "gas" and
20 pressure in the mid-epigastrium, but without dysphagia is suggestive of other gastrointestinal complaints that have been observed in previous HBOL studies. L-arginine was used as a therapeutic measure based on the concept that hemoglobin can interfere with endogenous nitric oxide
25 function (nitric oxide is integral in the relaxation of gastrointestinal smooth muscle, especially in the esophagus and intestines). L-arginine is the substrate upon which nitric oxide synthase produces nitric oxide.
Theoretically, if one has a reduction in nitric oxide from
30 the hemoglobin (perhaps binding of heme to nitric oxide), then administration of L-arginine might be of benefit. Apparently the subject did get marked by transient relief from his symptoms with the L-arginine for about two hours. This is not an unexpected finding because the plasma half-

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life of L-arginine is about an hour. Unfortunately, some of the side effects (nausea and vomiting) occurred and the infusion was stopped. We elected to give him a two doses of an anticholinergic, antispasmodic drug, hyoscyamine.

5 This apparently continued to reduce the symptoms of abdominal "gas" and pressure. The subject had no further complaints or sequelae.

In summary, HBOL was associated with improved oxygen delivery and utilization during exercise and at rest. HBOL 10 produced a similar spectrum of hemodynamic, safety laboratory results, pharmacokinetics and medical events to what has previously been observed. Intervention with L- arginine may produce a reversal of gastrointestinal symptoms, but its use was limited by nausea and vomiting. 15 However, the use of anticholinergic therapy might be of value in the treatment for the gastrointestinal symptoms that are encountered.

EQUIVALENTS

Those skilled in the art will recognize, or be able to 20 ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other such equivalents are intended to be encompassed by the following claims.

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CLAIMS

The invention claimed is:

1. A method for producing a purified hemoglobin product, comprising the steps of:
 - a) loading a hemoglobin solution onto an anion exchange chromatography column; and
 - b) injecting at least one tris(hydroxymethyl) aminomethane acetate buffer solution into the column, said buffer solution having a pH lower than that of the column, whereby a purified hemoglobin product elutes from the column.
2. The method of Claim 1, wherein at least two tris(hydroxymethyl) aminomethane acetate buffer solutions are injected sequentially into the column, each said buffer solution having a distinct pH, whereby the column is subjected to a stepped pH gradient.
3. The method of Claim 2, wherein the column is equilibrated with at least one of said tris(hydroxymethyl) aminomethane acetate buffer solutions prior to elution of the hemoglobin product.
4. The method of Claim 3, wherein the column is equilibrated at a pH in a range of between about 8.2 and about 8.6.
5. The method of Claim 4, wherein the column initially is equilibrated at a pH above about 8.7 prior to injecting said buffer solutions.

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6. The method of Claim 5, wherein tris(hydroxymethyl) aminomethane acetate buffer is employed to initially equilibrate said column.
7. The method of Claim 6, wherein the pH of initial equilibration is in a range of between about 8.7 and about 10.0.
8. The method of Claim 7, wherein the pH of initial equilibration is in a range of between about 8.7 and about 9.3
9. The method of Claim 8, wherein the pH of initial equilibration is in a range of between about 8.9 and about 9.1.
10. The method of Claim 4, wherein at least about eleven column void volumes of the buffer solution are injected into the column during said equilibration of the column.
11. The method of Claim 10, wherein said equilibration is at a pH in a range of between about 8.2 and about 8.4.
12. The method of Claim 11, wherein the hemoglobin product is eluted with a buffer at a pH in a range of between about 6.5 and about 7.5.
13. A method for producing a purified hemoglobin product, comprising the steps of:
 - a) loading a hemoglobin solution onto an anion exchange chromatography column, said loaded column initially being equilibrated to a pH greater than about 8.7; and

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- b) injecting at least one buffer solution into the column, said buffer solution having a pH lower than about 8.6, whereby a purified hemoglobin product elutes from the column.
- 14. The method of Claim 13, wherein said initial equilibration is at a pH in a range of between about 8.7 and about 10.0.
- 15. The method of Claim 14, wherein said initial equilibration is at a pH in a range of between about 8.7 and about 9.3.
- 16. The method of Claim 15, wherein said initial equilibration is at a pH in a range of between about 8.9 and about 9.1.
- 17. The method of Claim 16, wherein the column is equilibrated initially with tris(hydroxymethyl) aminomethane acetate.
- 18. The method of Claim 17, wherein at least two tris(hydroxymethyl) aminomethane acetate buffer solutions having a pH below about 8.6 are injected sequentially into the column, each said buffer solution having a distinct pH, whereby the column is subjected to a stepped pH gradient.
- 19. The method of Claim 18, wherein the column is equilibrated with at least one of said tris(hydroxymethyl) aminomethane acetate buffer solutions prior to elution of the hemoglobin product.

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20. The method of Claim 19, wherein the column is equilibrated at a pH in a range of between about 8.2 and about 8.6.
21. The method of Claim 20, wherein at least about eleven column void volumes of the buffer solution are injected into the column during said equilibration of the column.
22. The method of Claim 21, wherein said equilibration is at a pH in a range of between about 8.2 and about 8.4.
23. The method of Claim 22, wherein the hemoglobin product is eluted at a pH in a range of between about 6.5 and about 7.5.
24. A method for producing a purified hemoglobin product, comprising the steps of:
 - a) loading a hemoglobin solution onto an anion exchange chromatography column;
 - b) injecting to the column at least eleven column void volumes of an equilibrating buffer solution having a pH in a range of between about 8.2 and about 8.6; and
 - c) injecting into the column a buffer solution having a pH lower than that of the equilibrating buffer solution, whereby a purified hemoglobin product elutes from the column.
25. The method of Claim 24, wherein the equilibrating buffer solution has a pH of about 8.3.
26. The method of Claim 24, wherein the buffer solution is tris(hydroxymethyl) aminomethane acetate.

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27. The method of Claim 26, wherein at least two tris(hydroxymethyl) aminomethane acetate buffer solutions are injected sequentially into the column, each said buffer solution having a distinct pH, whereby the column is subjected to a stepped pH gradient.
28. The method of Claim 27, wherein the column initially is equilibrated at a pH above about 8.7 prior to injecting said buffer solutions.
29. The method of Claim 28, wherein tris(hydroxymethyl) aminomethane acetate buffer is employed to initially equilibrate said column.
30. The method of Claim 29, wherein the pH of initial equilibration is in a range of between about 8.7 and about 10.0.
31. The method of Claim 30, wherein the pH of initial equilibration is in a range of between about 8.7 and about 9.3.
32. The method of Claim 31, wherein the pH of initial equilibration is in a range of between about 8.9 and about 9.1.
33. The method of Claim 32, wherein the hemoglobin product is eluted at a pH in a range of between about 6.5 and about 7.5.
34. A method for producing a purified hemoglobin product, comprising the steps of:

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- a) loading a hemoglobin solution onto an anion exchange chromatography column, said loaded column initially being equilibrated to a pH greater than about 8.7;
- b) injecting into the column at least eleven column void volumes of an equilibrating buffer solution of tris(hydroxymethane) aminomethane acetate having a pH in a range of between about 8.2 and about 8.6; and
- c) injecting at least one tris(hydroxymethane) aminomethane acetate buffer solution into the column, said buffer solution having a pH lower than about 8.2, whereby a purified hemoglobin product elutes from the column.

35. The method of Claim 34, wherein tris(hydroxymethyl) aminomethane acetate buffer is employed to initially equilibrate said column.

36. The method of Claim 35, wherein the pH of initial equilibration is in a range of between about 8.7 and about 10.0.

37. The method of Claim 36, wherein the pH of initial equilibration is in a range of between about 8.7 and about 9.3

38. The method of Claim 37, wherein the pH of initial equilibration is in a range of between about 8.9 and about 9.1.

39. The method of Claim 38, wherein said equilibrating buffer solution has a pH in a range of between about 8.2 and about 8.4.

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40. The method of Claim 39, wherein the hemoglobin product is eluted with a buffer at a pH in a range of between about 6.5 and about 7.5.
41. The method of Claim 34, wherein the chromatography columns are packed with an anion exchange medium.
42. The method of Claim 41, wherein the anion exchange medium is selected from the group consisting of an amine- or ammonium-containing silica gel, alumina gel, titania gel, cross-linked dextran, agarose, a polyacrylamide, a polyhydroxyethyl-methacrylate or styrene divinylbenzene.
43. The method of Claim 42, wherein the anion exchange medium is an amine or ammonium containing silica gel.
44. The method of Claim 34, wherein the purified hemoglobin product includes less than 0.5 endotoxin units per milliliter, less than 3.3 nmoles/ml phospholipids and essentially no non-hemoglobin proteins.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/13922

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/805

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 29346 A (BIOPURE CORP) 26 September 1996 (1996-09-26) page 19, line 13 -page 22, line 5 page 56, line 25 -page 58, line 3 -----	1-44
A	WO 96 15151 A (SOMATOPEN INC) 23 May 1996 (1996-05-23) page 19, line 31 -page 20, line 10 -----	1-44
A	US 5 264 555 A (SHORR ROBERT G L ET AL) 23 November 1993 (1993-11-23) column 7, line 46 -column 11, line 23 -----	1-44



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter	Final Application No
	PCT/US 99/13922

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9629346	A 26-09-1996	US 5854209 A US 5840852 A US 5691452 A AU 705225 B AU 5322796 A CA 2215697 A EP 0815138 A JP 11502821 T US 5753616 A US 5895810 A		29-12-1998 24-11-1998 25-11-1997 20-05-1999 08-10-1996 26-09-1996 07-01-1998 09-03-1997 19-05-1998 20-04-1999
WO 9615151	A 23-05-1996	US 5840851 A AU 2478295 A CA 2203133 A EP 0792293 A		24-11-1998 06-06-1996 23-05-1996 03-09-1998
US 5264555	A 23-11-1993	AU 4638393 A EP 0654039 A WO 9401452 A		31-01-1994 24-05-1995 20-01-1994